

**INTERFERENCE INITIAL MEMORANDUM**

Count # \_\_\_\_\_

To the Board of Patent Appeals and Interferences:

**An interference is proposed involving the following 2 parties**

PARTY	APPLICATION NO.	FILING DATE	PATENT NO., IF ANY	ISSUE DATE, IF ANY
Junior Party Ni et al.	10/648,825	08/27/2003	N/A	N/A

If the involved is a patent, have its maintenance fees been paid? Yes ☐ No ☐ Not due yet ☒

Proposed priority benefit (list all intervening applications necessary for continuity):

COUNTRY	APPLICATION NO.	FILING DATE	PATENT NO., IF ANY	ISSUE DATE, IF ANY
USA	10/648,825	08/27/2003	N/A	N/A

The claim(s) of this party corresponding to Proposed Count 1:  
78-91, 134-145, 182-195, and 238-249

PATENTED OR PATENTABLE PENDING CLAIMS

UNPATENTABLE PENDING CLAIMS

The claim(s) of this party NOT corresponding to Proposed Count 1:  
None

PARTY	APPLICATION NO.	FILING DATE	PATENT NO., IF ANY	ISSUE DATE, IF ANY
Senior Party Adams et al.	10/052,798	11/02/2001	N/A	N/A

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USA	09/079,029	05/14/1998	6,342,369	01/29/2002

The claim(s) of this party corresponding to Proposed Count 1:  
59, 60, 65, 66, 69-74, 79, 125, 127-129, 133, 135-137, and 147-155

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- ☐ 1. Obtain all files listed above.

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- ☐ 3. If one of the involved files is a published application or a patent, check for compliance with 35 U.S.C. 135(b).
- ☐ 4. Obtain a certified copy of any foreign benefit documents where necessary (37 CFR 1.55(a)).
- ☐ 5. Discuss the proposed interference with an Interference Practice Specialist in your Technology Center.

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USA	60/148,939	08/13/1999	N/A	N/A
USA	60/133,238	05/07/1999	N/A	N/A
USA	60/132,498	05/04/1999	N/A	N/A
USA	09/042,583	03/17/1998	N/A	N/A

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USA	60/054,021	07/29/1997	N/A	N/A

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USA	60/040,846	03/17/1997	N/A	N/A

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## **APPENDIX B**

### **Pending Claims of U.S. Application No. 10/052,798 to Adams et al. (Claims 59, 60, 65, 66, 69-74, 79, 125, 127-129, 133, 135-137, and 147-155)**

59. A method of inducing apoptosis in mammalian cancer cells comprising exposing mammalian cancer cells to an effective amount of an Apo-2 agonist monoclonal antibody which (a) binds to Apo-2 polypeptide consisting of the contiguous amino acid residues 1 to 411 of SEQ ID NO:1 and (b) induces apoptosis in at least one type of mammalian cancer cell *in vivo* or *ex vivo*.

60. The method of claim 59 wherein said antibody comprises a single-chain antibody.

65. A method of inducing apoptosis in mammalian cancer cells comprising exposing mammalian cancer cells to an effective amount of an Apo-2 agonist monoclonal antibody which (a) binds to a soluble extracellular domain sequence of an Apo-2 polypeptide consisting of amino acids 54 to 182 of SEQ ID NO:1 and (b) induces apoptosis in at least one type of mammalian cancer cell *in vivo* or *ex vivo*.

66. A method of inducing apoptosis in mammalian cancer cells comprising exposing mammalian cancer cells to an effective amount of an Apo-2 agonist monoclonal antibody which (a) binds to a soluble extracellular domain sequence of an Apo-2 polypeptide consisting of amino acids 1 to 182 of SEQ ID NO:1 and (b) induces apoptosis in at least one type of mammalian cancer cell *in vivo* or *ex vivo*.

69. The method of claim 59, 65, or 66, wherein said antibody is a chimeric antibody.

70. The method of claim 59, 65, or 66, wherein said antibody is a humanized antibody.

71. The method of claim 59, 65, or 66, wherein said antibody is a human antibody.

72. The method of claim 59, 65, or 66, wherein said antibody comprises an Fab fragment.
73. The method of claim 59, 65, or 66, wherein said antibody comprises a scFv fragment.
74. The method of claim 59, 65, or 66, wherein said antibody comprises a F(ab')<sub>2</sub> fragment.
79. The method of claim 59, 65, or 66, wherein said antibody is fused to an epitope tag sequence.
125. A method of treating cancer comprising exposing mammalian cancer cells to an effective amount of an Apo-2 agonist monoclonal antibody which (a) binds to Apo-2 polypeptide consisting of the contiguous amino acid residues 1 to 411 of SEQ ID NO:1 and (b) induces apoptosis in said mammalian cancer cell *in vivo* or *ex vivo*.
127. The method of claim 125 wherein said agonist antibody is a chimeric antibody.
128. The method of claim 125 wherein said agonist antibody is a humanized antibody.
129. The method of claim 125 wherein said agonist antibody is a human antibody.
133. A method of treating cancer comprising exposing mammalian cancer cells to an effective amount of an Apo-2 agonist monoclonal antibody which (a) binds to a soluble extracellular domain sequence of an Apo-2 polypeptide which consists of amino acid residues 54 to 182 of SEQ ID NO:1 and (b) induces apoptosis in said mammalian cancer cell *in vivo* or *ex vivo*.
135. The method of claim 133 wherein said agonist antibody is a chimeric antibody.
136. The method of claim 133 wherein said agonist antibody is a humanized antibody.
137. The method of claim 133 wherein said agonist antibody is a human antibody.



147. A method of treating cancer comprising exposing mammalian cancer cells to an effective amount of an Apo-2 agonist monoclonal antibody which (a) binds to a soluble extracellular domain sequence of an Apo-2 polypeptide consisting of amino acid residues 1 to 182 of SEQ ID NO:1 and (b) induces apoptosis in said mammalian cancer cell *in vivo* or *ex vivo*.

148. The method of claim 147 wherein said agonist antibody is a chimeric antibody.

149. The method of claim 147 wherein said agonist antibody is a humanized antibody.

150. The method of claim 147 wherein said agonist antibody is a human antibody.

151. The method of claim 125, 133, or 147, wherein said antibody comprises an Fab fragment.

152. The method of claim 125, 133, or 147, wherein said antibody comprises a scFv fragment.

153. The method of claim 125, 133, or 147, wherein said antibody comprises a F(ab')<sub>2</sub> fragment.

154. The method of claim 125, 133, or 147, wherein said antibody is fused to an epitope tag sequence.

155. The method of claim 125, 133, or 147, wherein said mammalian cancer cells are exposed to chemotherapy or radiation therapy.

## APPENDIX C

### **Pending Claims of U.S. Application No. 10/648,825 to Ni et al. (Claims 78-91, 134-145, 182-195, and 238-249)**

78. A method of inducing apoptosis of a DR5-expressing cell, comprising contacting said cell with an agonist antibody or fragment thereof that specifically binds to a polypeptide consisting of amino acids 1 to 133 of SEQ ID NO:2.

79. The method of claim 78 which is *in vitro*.

80. The method of claim 78 which is *in vivo*.

81. The method of claim 78, wherein the polypeptide is glycosylated.

82. The method of claim 78, wherein said antibody or fragment thereof is polyclonal.

83. The method of claim 78, wherein said antibody or fragment thereof is monoclonal.

84. The method of claim 78, wherein said antibody or fragment thereof is selected from the group consisting of:

(a) a Fab fragment; and

(b) a F(ab')<sub>2</sub> fragment.

85. The method of claim 78, wherein said antibody or fragment thereof is labeled.

86. The method of claim 85, wherein said label is selected from the group consisting of:

(a) an enzyme;

(b) a fluorescent label; and

(c) a radioisotope.

87. The method of claim 78, wherein said antibody or fragment thereof specifically binds to said polypeptide in a Western blot.

88. The method of claim 78, wherein said antibody or fragment thereof specifically binds to said polypeptide in an ELISA.

89. The method of claim 78, further comprising contacting said cell with a compound that potentiates apoptosis selected from the group consisting of:

- (a) TRAIL; and
- (b) a chemotherapeutic drug.

90. The method of claim 89, wherein said compound is TRAIL.

91. The method of claim 89, wherein said compound is a chemotherapeutic drug.

134. A method of treating cancer, comprising administering to a patient an agonist antibody or fragment thereof that specifically binds to a polypeptide consisting of amino acids 1 to 133 of SEQ ID NO:2, wherein said antibody or fragment thereof is administered in an amount sufficient to induce apoptosis of a DR5-expressing cancer cell.

135. The method of claim 134, wherein the polypeptide is glycosylated.

136. The method of claim 134, wherein said antibody or fragment thereof is polyclonal.

137. The method of claim 134, wherein said antibody or fragment thereof is monoclonal.

138. The method of claim 134, wherein said antibody or fragment thereof is selected from the group consisting of:

- (a) a Fab fragment; and
- (b) a F(ab')<sub>2</sub> fragment.

139. The method of claim 134, wherein said antibody or fragment thereof is labeled.

140. The method of claim 139, wherein said label is selected from the group consisting of:

- (a) an enzyme;
- (b) a fluorescent label; and
- (c) a radioisotope.

141. The method of claim 134, wherein said antibody or fragment thereof specifically binds to said polypeptide in a Western blot.

142. The method of claim 134, wherein said antibody or fragment thereof specifically binds to said polypeptide in an ELISA.

143. The method of claim 134, further comprising contacting said cell with a compound that potentiates apoptosis selected from the group consisting of:

- (a) TRAIL; and
- (b) a chemotherapeutic drug.

144. The method of claim 143, wherein said compound is TRAIL.

145. The method of claim 143, wherein said compound is a chemotherapeutic drug.

182. A method of inducing apoptosis of a DR5-expressing cell, comprising contacting said cell with an antibody or fragment thereof that specifically binds to a polypeptide consisting of amino acids 1 to 133 of SEQ ID NO:2.

183. The method of claim 182 which is *in vitro*.

184. The method of claim 182 which is *in vivo*.

185. The method of claim 182, wherein the polypeptide is glycosylated.

186. The method of claim 182, wherein said antibody or fragment thereof is polyclonal.

187. The method of claim 182, wherein said antibody or fragment thereof is monoclonal.

188. The method of claim 182, wherein said antibody or fragment thereof is selected from the group consisting of:

- (a) a Fab fragment; and
- (b) a F(ab')<sub>2</sub> fragment.

189. The method of claim 182, wherein said antibody or fragment thereof is labeled.

190. The method of claim 189, wherein said label is selected from the group consisting of:

- (a) an enzyme;
- (b) a fluorescent label; and
- (c) a radioisotope.

191. The method of claim 182, wherein said antibody or fragment thereof specifically binds to said polypeptide in a Western blot.

192. The method of claim 182, wherein said antibody or fragment thereof specifically binds to said polypeptide in an ELISA.

193. The method of claim 182, further comprising contacting said cell with a compound that potentiates apoptosis selected from the group consisting of:

- (a) TRAIL; and
- (b) a chemotherapeutic drug.

194. The method of claim 193, wherein said compound is TRAIL.

195. The method of claim 193, wherein said compound is a chemotherapeutic drug.

238. A method of treating cancer, comprising administering to a patient an antibody or fragment thereof that specifically binds to a polypeptide consisting of amino acids 1 to 133 of

SEQ ID NO:2, wherein said antibody or fragment thereof is administered in an amount sufficient to induce apoptosis of a DR5-expressing cancer cell.

239. The method of claim 238, wherein the polypeptide is glycosylated.

240. The method of claim 238, wherein said antibody or fragment thereof is polyclonal.

241. The method of claim 238, wherein said antibody or fragment thereof is monoclonal.

242. The method of claim 238, wherein said antibody or fragment thereof is selected from the group consisting of:

- (a) a Fab fragment; and
- (b) a F(ab')<sub>2</sub> fragment.

243. The method of claim 238, wherein said antibody or fragment thereof is labeled.

244. The method of claim 243, wherein said label is selected from the group consisting of:

- (a) an enzyme;
- (b) a fluorescent label; and
- (c) a radioisotope.

245. The method of claim 238, wherein said antibody or fragment thereof specifically binds to said polypeptide in a Western blot.

246. The method of claim 238, wherein said antibody or fragment thereof specifically binds to said polypeptide in an ELISA.

247. The method of claim 238, further comprising contacting said cell with a compound that potentiates apoptosis selected from the group consisting of:

- (a) TRAIL; and
- (b) a chemotherapeutic drug.

248. The method of claim 247, wherein said compound is TRAIL.

249. The method of claim 247, wherein said compound is a chemotherapeutic drug.

## APPENDIX D

### Comparison of Amino Acid Residues 54-182 of Applicants' SEQ ID NO:1 and Amino Acid Residues 3-131 of Ni's '825 Application SEQ ID NO:2

Applicants' SEQ ID NO:1	54	55	56	57	58	59	60	61	62	63	64	65
Ni's '825 Application SEQ ID NO:2	3	4	5	6	7	8	9	10	11	12	13	14
<b>Amino Acid</b>	<b>Ala</b>	<b>Leu</b>	<b>Ile</b>	<b>Thr</b>	<b>Gln</b>	<b>Gln</b>	<b>Asp</b>	<b>Leu</b>	<b>Ala</b>	<b>Pro</b>	<b>Gln</b>	<b>Gln</b>

Applicants' SEQ ID NO:1	66	67	68	69	70	71	72	73	74	75	76	77
Ni's '825 Application SEQ ID NO:2	15	16	17	18	19	20	21	22	23	24	25	26
<b>Amino Acid</b>	<b>Arg</b>	<b>Ala</b>	<b>Ala</b>	<b>Pro</b>	<b>Gln</b>	<b>Gln</b>	<b>Lys</b>	<b>Arg</b>	<b>Ser</b>	<b>Ser</b>	<b>Pro</b>	<b>Ser</b>

Applicants' SEQ ID NO:1	78	79	80	81	82	83	84	85	86	87	88	89
Ni's '825 Application SEQ ID NO:2	27	28	29	30	31	32	33	34	35	36	37	38
<b>Amino Acid</b>	<b>Glu</b>	<b>Gly</b>	<b>Leu</b>	<b>Cys</b>	<b>Pro</b>	<b>Pro</b>	<b>Gly</b>	<b>His</b>	<b>His</b>	<b>Ile</b>	<b>Ser</b>	<b>Glu</b>

Applicants' SEQ ID NO:1	90	91	92	93	94	95	96	97	98	99	100	101
Ni's '825 Application SEQ ID NO:2	39	40	41	42	43	44	45	46	47	48	49	50
<b>Amino Acid</b>	<b>Asp</b>	<b>Gly</b>	<b>Arg</b>	<b>Asp</b>	<b>Cys</b>	<b>Ile</b>	<b>Ser</b>	<b>Cys</b>	<b>Lys</b>	<b>Tyr</b>	<b>Gly</b>	<b>Gln</b>

Applicants' SEQ ID NO:1	102	103	104	105	106	107	108	109	110	111	112	113
Ni's '825 Application SEQ ID NO:2	51	52	53	54	55	56	57	58	59	60	61	62
<b>Amino Acid</b>	<b>Asp</b>	<b>Tyr</b>	<b>Ser</b>	<b>Thr</b>	<b>His</b>	<b>Trp</b>	<b>Asn</b>	<b>Asp</b>	<b>Leu</b>	<b>Leu</b>	<b>Phe</b>	<b>Cys</b>



Applicants' SEQ ID NO:1	114	115	116	117	118	119	120	121	122	123	124	125
Ni's '825 Application SEQ ID NO:2	63	64	65	66	67	68	69	70	71	72	73	74
<b>Amino Acid</b>	<b>Leu</b>	<b>Arg</b>	<b>Cys</b>	<b>Thr</b>	<b>Arg</b>	<b>Cys</b>	<b>Asp</b>	<b>Ser</b>	<b>Gly</b>	<b>Glu</b>	<b>Val</b>	<b>Glu</b>

Applicants' SEQ ID NO:1	126	127	128	129	130	131	132	133	134	135	136	137
Ni's '825 Application SEQ ID NO:2	75	76	77	78	79	80	81	82	83	84	85	86
<b>Amino Acid</b>	<b>Leu</b>	<b>Ser</b>	<b>Pro</b>	<b>Cys</b>	<b>Thr</b>	<b>Thr</b>	<b>Thr</b>	<b>Arg</b>	<b>Asn</b>	<b>Thr</b>	<b>Val</b>	<b>Cys</b>

Applicants' SEQ ID NO:1	138	139	140	141	142	143	144	145	146	147	148	149
Ni's '825 Application SEQ ID NO:2	87	88	89	90	91	92	93	94	95	96	97	98
<b>Amino Acid</b>	<b>Gln</b>	<b>Cys</b>	<b>Glu</b>	<b>Glu</b>	<b>Gly</b>	<b>Thr</b>	<b>Phe</b>	<b>Arg</b>	<b>Glu</b>	<b>Glu</b>	<b>Asp</b>	<b>Ser</b>

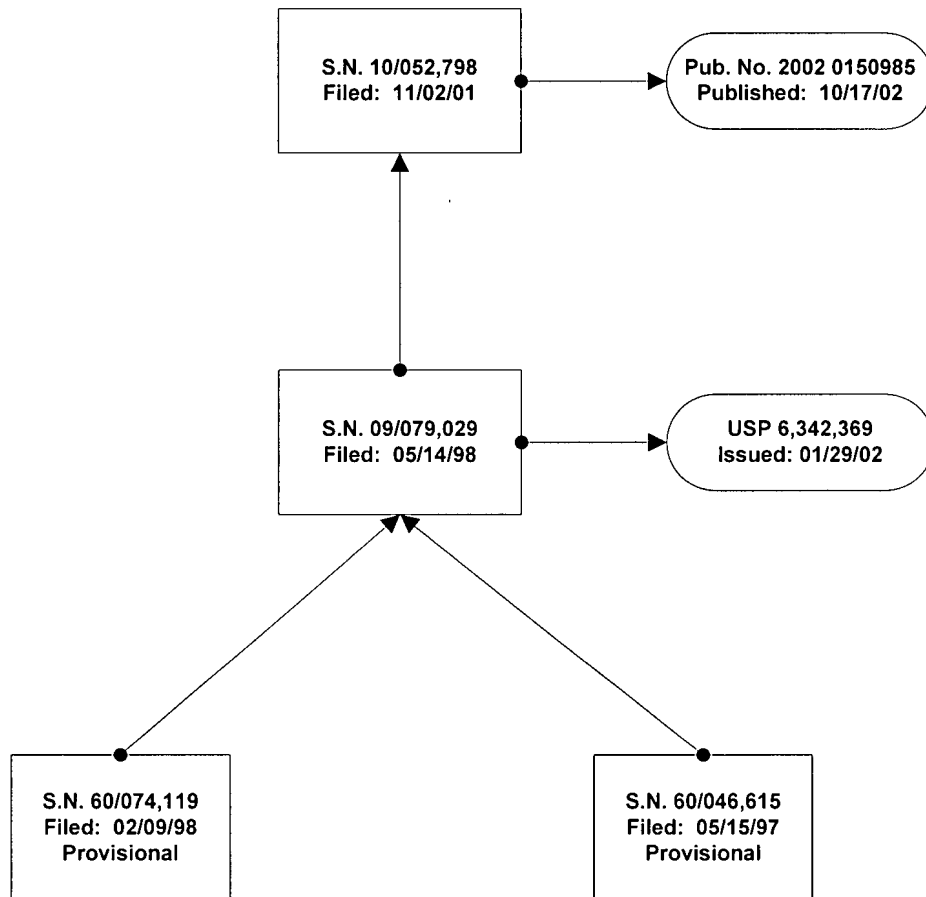
Applicants' SEQ ID NO:1	150	151	152	153	154	155	156	157	158	159	160	161
Ni's '825 Application SEQ ID NO:2	99	100	101	102	103	104	105	106	107	108	109	110
<b>Amino Acid</b>	<b>Pro</b>	<b>Glu</b>	<b>Met</b>	<b>Cys</b>	<b>Arg</b>	<b>Lys</b>	<b>Cys</b>	<b>Arg</b>	<b>Thr</b>	<b>Gly</b>	<b>Cys</b>	<b>Pro</b>

Applicants' SEQ ID NO:1	162	163	164	165	166	167	168	169	170	171	172	173
Ni's '825 Application SEQ ID NO:2	111	112	113	114	115	116	117	118	119	120	121	122
<b>Amino Acid</b>	<b>Arg</b>	<b>Gly</b>	<b>Met</b>	<b>Val</b>	<b>Lys</b>	<b>Val</b>	<b>Gly</b>	<b>Asp</b>	<b>Cys</b>	<b>Thr</b>	<b>Pro</b>	<b>Trp</b>

Applicants' SEQ ID NO:1	174	175	176	177	178	179	180	181	182
Ni's '825 Application SEQ ID NO:2	123	124	125	126	127	128	129	130	131
<b>Amino Acid</b>	<b>Ser</b>	<b>Asp</b>	<b>Ile</b>	<b>Glu</b>	<b>Cys</b>	<b>Val</b>	<b>His</b>	<b>Lys</b>	<b>Glu</b>

## APPENDIX E

### Earlier-Filed Adams *et al.* Applications



## APPENDIX F

### Constructive Reduction to Practice of Proposed Count in 60/046,615 Specification

<b>Proposed Count</b>	<b>Support in Applicants' 60/046,615 Specification</b>
65. A method of inducing apoptosis in mammalian cancer cells comprising	<p>"‘Biologically active’ and ‘desired biological activity’ for the purposes herein means having the ability to modulate apoptosis (either in an agonistic or stimulating manner or in an antagonistic or blocking manner) in at least one type of mammalian cell <i>in vivo</i> or <i>ex vivo</i>." ADE-18, P. 16, l. 34 – P. 17, l. 3.</p> <p>"The terms ‘apoptosis’ and ‘apoptotic activity’ are used in a broad sense and refer to the orderly or controlled form of cell death in mammals that is typically accompanied by one or more characteristic cell changes, including condensation of cytoplasm, loss of plasma membrane microvilli, segmentation of the nucleus, degradation of chromosomal DNA or loss of mitochondrial function. This activity can be determined and measured, for instance, by cell viability assays, FACS analysis or DNA electrophoresis, all of which are known in the art." ADE-18, P. 17, ll. 4-12.</p> <p>"The term ‘mammal’ as used herein refers to any mammal classified as a mammal, including humans, cows, horses, dogs and cats. In a preferred embodiment of the invention, the mammal is a human." ADE-18, P. 17, ll. 16-19.</p> <p>"Apo-2, as disclosed in the present specification, can be employed therapeutically to induce apoptosis in mammalian cells. This therapy can be accomplished for instance, using <i>in vivo</i> or <i>ex vivo</i> gene therapy techniques and includes the use of the death domain sequences disclosed herein. The Apo-2 chimeric molecules (including the chimeric molecules containing an extracellular domain sequence of Apo-2) comprising immunoglobulin sequences can also be employed therapeutically to inhibit apoptosis or NF-κB induction by Apo-2L or by another ligand that Apo-2 binds to." ADE-18, P. 45, ll. 2-10.</p> <p>"The Apo-2 antibodies of the invention have therapeutic utility. Agonistic Apo-2 antibodies, for instance, may be employed to activate or stimulate apoptosis in cancer cells." ADE-18, P. 56, ll. 21-23.</p>
exposing mammalian cancer cells to an effective amount of an Apo-2 agonist monoclonal antibody	<p>"In another embodiment, the invention provides an antibody which specifically binds to Apo-2. The antibody may be an agonistic, antagonistic or neutralizing antibody." ADE-18,</p>

Proposed Count	Support in Applicants' 60/046,615 Specification
which	<p data-bbox="659 243 829 281"><b>P. 10, ll. 3-5.</b></p> <p data-bbox="659 317 1430 428">“A further embodiment of the invention provides articles of manufacture and kits that include Apo-2 or Apo-2 antibodies.” <b>ADE-18, P. 10, ll. 8-9.</b></p> <p data-bbox="659 464 1430 646">“The term ‘antibody’ is used in the broadest sense and specifically covers single anti-Apo-2 monoclonal antibodies (including agonist, antagonist, and neutralizing antibodies) and anti-Apo-2 antibody compositions with polyepitopic specificity.</p> <p data-bbox="659 653 1430 1052">The term ‘monoclonal antibody’ as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, <i>i.e.</i>, the individual antibodies comprising the population are identical except for possible naturally-occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen.</p> <p data-bbox="659 1058 1430 1528">The monoclonal antibodies herein include hybrid and recombinant antibodies produced by splicing a variable (including hypervariable) domain of an anti-Apo-2 antibody with a constant domain (<i>e.g.</i> ‘humanized’ antibodies), or a light chain with a heavy chain, or a chain from one species with a chain from another species, or fusions with heterologous proteins, regardless of species of origin or immunoglobulin class or subclass designation, as well as antibody fragments (<i>e.g.</i>, Fab, F(ab')<sub>2</sub>, and Fv), so long as they exhibit the desired biological activity. See, <i>e.g.</i> U.S. Pat. No. 4,816,567 and Mage et al., in <u>Monoclonal Antibody Production Techniques and Applications</u>, pp.79-97 (Marcel Dekker, Inc.: New York, 1987).</p> <p data-bbox="659 1535 1430 1858">Thus, the modifier ‘monoclonal’ indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler and Milstein, <u>Nature</u>, 256:495 (1975), or may be made by recombinant DNA methods such as described in U.S. Pat.</p>

Proposed Count	Support in Applicants' 60/046,615 Specification
	<p>No. 4,816,567. The 'monoclonal antibodies' may also be isolated from phage libraries generated using the techniques described in McCafferty et al., <u>Nature</u>, 348:552-554 (1990), for example.</p> <p>'Humanized' forms of non-human (<i>e.g.</i> murine) antibodies are specific chimeric immunoglobulins, immunoglobulin chains, or fragments thereof (such as Fv, Fab, Fab', F(ab')<sub>2</sub> or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat, or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, the humanized antibody may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region or domain (Fc), typically that of a human immunoglobulin." ADE-18, P. 15, l. 7 – P. 16, l. 33.</p> <p>"A description follows as to how Apo-2, as well as Apo-2 chimeric molecules and anti-Apo-2 antibodies, may be prepared." ADE-18, P. 17, ll. 30-31.</p> <p>"Preferably, the Ig is a human immunoglobulin when the chimeric molecule is intended for <i>in vivo</i> therapy for humans." ADE-18, P. 44, ll. 11-12.</p> <p>"Apo-2 preparations are also useful in generating antibodies..." ADE-18, P. 45, ll. 22-23.</p>

Proposed Count	Support in Applicants' 60/046,615 Specification
	<p data-bbox="656 247 1435 394">“Modified forms of the Apo-2, such as the Apo-2-IgG chimeric molecules (immunoadhesins) described above, can be used as immunogens in producing anti-Apo-2 antibodies.” <b>ADE-18, P. 45, ll. 29-31.</b></p> <p data-bbox="656 436 1386 615">“The present invention further provides anti-Apo-2 antibodies. Antibodies against Apo-2 may be prepared as follows. Exemplary antibodies include polyclonal, monoclonal, humanized, bispecific, and heteroconjugate antibodies.” <b>ADE-18, P. 47, ll. 26-29.</b></p> <p data-bbox="656 657 1435 1014">“The Apo-2 antibodies may, alternatively, be monoclonal antibodies. Monoclonal antibodies may be prepared using hybridoma methods, such as those described by Kohler and Milstein, <u>supra</u>. In a hybridoma method, a mouse, hamster, or other appropriate host animal, is typically immunized (such as described above) with an immunizing agent to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the immunizing agent. Alternatively, the lymphocytes may be immunized <i>in vitro</i>.” <b>ADE-18, P. 48, ll. 21-29.</b></p> <p data-bbox="656 1056 1435 1350">“The monoclonal antibodies may also be made by recombinant DNA methods, such as those described in U.S. Patent No. 4,816,567. DNA encoding the monoclonal antibodies of the invention can be readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies).” <b>ADE-18, P. 50, ll. 15-21.</b></p> <p data-bbox="656 1392 1419 1497">“The antibodies may be monovalent antibodies. Methods for preparing monovalent antibodies are well known in the art.” <b>ADE-18, P. 51, ll. 2-3.</b></p> <p data-bbox="656 1539 1354 1644">“The Apo-2 antibodies of the invention may further comprise humanized antibodies or human antibodies.” <b>ADE-18, P. 51, ll. 33-34.</b></p> <p data-bbox="656 1686 1403 1749">“Methods for humanizing non-human antibodies are well known in the art.” <b>ADE-18, P. 52, ll. 24-25.</b></p> <p data-bbox="656 1791 1386 1854">“The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is</p>

Proposed Count	Support in Applicants' 60/046,615 Specification
(a) binds to a soluble extracellular domain sequence of an Apo-2	<p>very important in order to reduce antigenicity. According to the 'best-fit' method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody [Sims et al., <i>J. Immunol.</i>, 151:2296 (1993); Chothia and Lesk, <i>J. Mol. Biol.</i>, 196:901 (1987)]." <b>ADE-18, P.53, ll. 7-15.</b></p>
	<p>"It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three dimensional models of the parental and humanized sequences. Three dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art." <b>ADE-18, P. 53, ll. 21-28.</b></p>
	<p>"Bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens. In the present case, one of the binding specificities is for the Apo-2, the other one is for any other antigen, and preferably for a cell-surface protein or receptor or receptor subunit.</p> <p>Methods for making bispecific antibodies are known in the art." <b>ADE-18, P. 54, ll. 26-33.</b></p>
	<p>"The Apo-2 antibodies of the invention have therapeutic utility. Agonistic Apo-2 antibodies, for instance, may be employed to activate or stimulate apoptosis in cancer cells." <b>ADE-18, P. 56, ll. 21-23.</b></p>
	<p>"In a further embodiment of the invention, there are provided articles of manufacture and kits containing Apo-2 or Apo-2 antibodies which can be used, for instance, for the therapeutic or non-therapeutic applications described above." <b>ADE-18, P. 57, ll. 25-28.</b></p>
	<p>"In another embodiment, the invention provides an isolated extracellular domain (ECD) sequence of Apo-2. Optionally,</p>

Proposed Count	Support in Applicants' 60/046,615 Specification
<p>polypeptide consisting of amino acids 54 to 182 SEQ ID NO:1 and</p>	<p>the isolated extracellular domain sequence comprises amino acid residues 54 to 182 of FIG. 1 (SEQ ID NO:1)." <b>ADE-18, P. 8, ll. 31-34.</b></p> <p>"FIG. 2A shows the derived amino acid sequence of a native sequence human Apo-2--the putative signal sequence is underlined, the putative transmembrane domain is boxed, and the putative death domain sequence is dash underlined. The cysteines of the two cysteine-rich domains are individually underlined." <b>ADE-18, P. 10, ll. 14-18.</b></p> <p>"The 'Apo-2 extracellular domain' or 'Apo-2 ECD' refers to a form of Apo-2 which is essentially free of the transmembrane and cytoplasmic domains of Apo-2. Ordinarily, Apo-2 ECD will have less than 1% of such transmembrane and/or cytoplasmic domains and preferably, will have less than 0.5% of such domains. Optionally, Apo-2 ECD will comprise amino acid residues 54 to 182 of FIG. 1 (SEQ ID NO:1) or amino acid residues 1 to 182 of FIG. 1 (SEQ ID NO:1)." <b>ADE-18, P. 12, ll. 15-22.</b></p> <p>"In general, the signal sequence may be a component of the vector, or it may be a part of the Apo-2 DNA that is inserted into the vector. The heterologous signal sequence selected preferably is one that is recognized and processed (i.e., cleaved by a signal peptidase) by the host cell...In mammalian cell expression the native Apo-2 presequence that normally directs insertion of Apo-2 in the cell membrane of human cells in vivo is satisfactory, although other mammalian signal sequences may be used to direct secretion of the protein, such as signal sequences from secreted polypeptides of the same or related species, as well as viral secretory leaders, for example, the herpes simplex glycoprotein D signal." <b>ADE-18, P. 21, ll. 12-32.</b></p> <p>"The entire nucleotide sequence of Apo-2 is shown in Figure 1 (SEQ ID NO:2). Clone 27868 (also referred to as pRK5-Apo-2 deposited as ATCC _____, as indicated below) contains a single open reading frame with an apparent translational initiation site at nucleotide positions 140-142 [Kozak et al., <i>supra</i>] and ending at the stop codon found at nucleotide positions 1373-1375 (Fig. 1; SEQ ID NO:2). The predicted polypeptide precursor is 411 amino acids long, a type I transmembrane protein, and has a</p>



Proposed Count	Support in Applicants' 60/046,615 Specification
(b) induces apoptosis in at least one type of mammalian cancer cell <i>in vivo</i> or <i>ex vivo</i> .	<p>calculated molecular weight of approximately 45 kDa. Hydropathy analysis (not shown) suggested the presence of a signal sequence (residues 1-53), followed by an extracellular domain (residues 54-182), a transmembrane domain (residues 183-208), and an intracellular domain (residues 209-411) (Fig. 2A; SEQ ID NO:1). N-terminal amino acid sequence analysis of Apo-2-IgG expressed in 293 cells showed that the mature polypeptide starts at amino acid residue 54, indicating that the actual signal sequence comprises residues 1-53.” <b>ADE-18, P. 61, ll. 10-25.</b></p> <p>“(The Apo-2 ECD construct included residues 183 and 184 shown in Figure. 1 to provide flexibility at the junction, even though residues 183 and 184 are predicted to be in the transmembrane region).” <b>ADE-18, P. 62, ll. 21-24.</b></p> <p>“A soluble Apo-2 ECD immunoadhesin construct was prepared. The Apo-2 ECD (amino acids 1-184 shown in Fig. 1) was fused to the hinge and Fc region of human immunoglobulin G<sub>1</sub> heavy chain in pRK5 as described previously [Ashkenazi et al., <u>Proc. Natl. Acad. Sci.</u>, 88:10535-10539 (1991)]. The immunoadhesin was expressed by transient transfection into human 293 cells and purified from cell supernatants by protein A affinity chromatography, as described by Ashkenazi et al., <u>supra</u>.” <b>ADE-18, P. 62, l. 34 - P. 63, l. 6.</b></p>
	<p>“The Apo-2 antibodies of the invention have therapeutic utility. Agonistic Apo-2 antibodies, for instance, may be employed to activate or stimulate apoptosis in cancer cells.” <b>ADE-18, P. 56, ll. 21-23.</b></p>

## APPENDIX G

### Constructive Reduction to Practice of Proposed Count in 60/074,119 Specification

Proposed Count	Support in Applicants' 60/074,119 Specification
65. A method of inducing apoptosis in mammalian cancer cells comprising	<p>“‘Biologically active’ and ‘desired biological activity’ for the purposes herein mean having the ability to modulate apoptosis (either in an agonistic or stimulating manner or in an antagonistic or blocking manner) in at least one type of mammalian cell <i>in vivo</i> or <i>ex vivo</i>.” ADE-33, P. 15, ll. 30-34.</p> <p>“The terms ‘apoptosis’ and ‘apoptotic activity’ are used in a broad sense and refer to the orderly or controlled form of cell death in mammals that is typically accompanied by one or more characteristic cell changes, including condensation of cytoplasm, loss of plasma membrane microvilli, segmentation of the nucleus, degradation of chromosomal DNA or loss of mitochondrial function. This activity can be determined and measured, for instance, by cell viability assays, FACS analysis or DNA electrophoresis, all of which are known in the art.” ADE-33, P. 15, ll. 35-42.</p> <p>“The term ‘mammal’ as used herein refers to any mammal classified as a mammal, including humans, cows, horses, dogs and cats. In a preferred embodiment of the invention, the mammal is a human.” ADE-33, P. 16, ll. 8-10.</p> <p>“Apo-2, as disclosed in the present specification, can be employed therapeutically to induce apoptosis in mammalian cells. This therapy can be accomplished for instance, using <i>in vivo</i> or <i>ex vivo</i> gene therapy techniques and includes the use of the death domain sequences disclosed herein. The Apo-2 chimeric molecules (including the chimeric molecules containing an extracellular domain sequence of Apo-2) comprising immunoglobulin sequences can also be employed therapeutically to inhibit apoptosis or NF-κB induction by Apo-2L or by another ligand that Apo-2 binds to.” ADE-33, P. 42, ll. 10-18.</p> <p>“The Apo-2 antibodies of the invention have therapeutic utility. Agonistic Apo-2 antibodies, for instance, may be employed to activate or stimulate apoptosis in cancer cells. The Apo-2 antibodies of the invention may also be useful in enhancing immune-mediated cell death in cells expressing Apo-2, for instance, through complement fixation or ADCC.” ADE-33, P. 53, ll. 16-21.</p>

Proposed Count	Support in Applicants' 60/074,119 Specification
<p>exposing mammalian cancer cells to an effective amount of an Apo-2 agonist monoclonal antibody which</p>	<p>“In another embodiment, the invention provides an antibody which specifically binds to Apo-2. The antibody may be an agonistic, antagonistic or neutralizing antibody. Dimeric molecules, in particular homodimeric molecules, comprising Apo-2 antibody are also provided.” <b>ADE-33, P. 9, ll. 18-21.</b></p> <p>“A further embodiment of the invention provides articles of manufacture and kits that include Apo-2 or Apo-2 antibodies.” <b>ADE-33, P. 9, ll. 24-25.</b></p> <p>“Figure 7 shows the FACS analysis of an Apo-2 antibody, 3F11.39.7 (illustrated by the bold lines) as compared to IgG controls (dotted lines). The 3F11.39.7 antibody recognized the Apo-2 receptor expressed in human 9D cells.” <b>ADE-33, P. 10, ll. 28-31.</b></p> <p>“Figure 8 is a graph showing percent (%) apoptosis induced in 9D cells by Apo-2 antibody 3F11.39.7, in the absence of goat anti-mouse IgG Fc.” <b>ADE-33, P. 10, ll. 32-34.</b></p> <p>“Figure 9 is a bar diagram showing percent (%) apoptosis, as compared to Apo-2L, in 9D cells by Apo-2 antibody 3F11.39.7 in the presence or absence of goat anti-mouse IgG Fc.” <b>ADE-33, P. 10, ll. 35-37.</b></p> <p>“Figure 11 is a graph showing results of an ELISA testing binding of Apo-2 antibody 3F11.39.7 to Apo-2 and to other known Apo-2L receptors referred to as DR4, DcR1, and DcR2.” <b>ADE-33, P. 10, l. 40 - P. 11, l. 5.</b></p> <p>“The term ‘antibody’ is used in the broadest sense and specifically covers anti-Apo-2 monoclonal antibodies (including agonist, antagonist, and blocking or neutralizing antibodies) and anti-Apo-2 antibody compositions with polyepitopic specificity.</p> <p>The term ‘monoclonal antibody’ as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, <i>i.e.</i>, the individual antibodies comprising the population are identical except for possible naturally-occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in</p>

Proposed Count	Support in Applicants' 60/074,119 Specification
	<p>contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen.</p> <p>The monoclonal antibodies herein include hybrid and recombinant antibodies produced by splicing a variable (including hypervariable) domain of an anti-Apo-2 antibody with a constant domain, or a light chain with a heavy chain, or a chain from one species with a chain from another species, or fusions with heterologous proteins, regardless of species of origin or immunoglobulin class or subclass designation, as well as antibody fragments (<i>e.g.</i>, Fab, F(ab')<sub>2</sub>, and Fv), so long as they exhibit the desired biological activity. See, <i>e.g.</i> U.S. Pat. No. 4,816,567 and Mage et al., in <u>Monoclonal Antibody Production Techniques and Applications</u>, pp.79-97 (Marcel Dekker, Inc.: New York, 1987).</p> <p>Thus, the modifier 'monoclonal' indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler and Milstein, <u>Nature</u>, 256:495 (1975), or may be made by recombinant DNA methods such as described in U.S. Pat. No. 4,816,567. The 'monoclonal antibodies' may also be isolated from phage libraries generated using the techniques described in McCafferty et al., <u>Nature</u>, 348:552-554 (1990), for example.</p> <p>'Humanized' forms of non-human (<i>e.g.</i> murine) antibodies are specific chimeric immunoglobulins, immunoglobulin chains, or fragments thereof (such as Fv, Fab, Fab', F(ab')<sub>2</sub> or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat, or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, the</p>

Proposed Count	Support in Applicants' 60/074,119 Specification
	<p>humanized antibody may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region or domain (Fc), typically that of a human immunoglobulin." ADE-33, P. 14, l. 10 - P. 15, l. 29.</p> <p>"The present invention provides newly identified and isolated Apo-2 polypeptides and Apo-2 antibodies. In particular, Applicants have identified and isolated various human Apo-2 polypeptides. The properties and characteristics of some of these Apo-2 polypeptides and anti-Apo-2 antibodies are described in further detail in the Examples below." ADE-33, P. 16, l. 13-18.</p> <p>"A description follows as to how Apo-2, as well as Apo-2 chimeric molecules and anti-Apo-2 antibodies, may be prepared." ADE-33, P. 16, ll. 21-22.</p> <p>"Preferably, the Ig is a human immunoglobulin when the chimeric molecule is intended for <i>in vivo</i> therapy for humans." ADE-33, P. 41, ll. 23-25.</p> <p>"Apo-2 preparations are also useful in generating antibodies..." ADE-33, P. 42, ll. 29-30.</p> <p>"Modified forms of the Apo-2, such as the Apo-2-IgG chimeric molecules (immunoadhesins) described above, can be used as immunogens in producing anti-Apo-2 antibodies." ADE-33, P. 42, ll. 35-37.</p> <p>"The present invention further provides anti-Apo-2 antibodies. Antibodies against Apo-2 may be prepared as follows. Exemplary antibodies include polyclonal, monoclonal, humanized, bispecific, and heteroconjugate antibodies." ADE-33, P. 44, ll. 24-27.</p>

Proposed Count	Support in Applicants' 60/074,119 Specification
	<p>“The Apo-2 antibodies may, alternatively, be monoclonal antibodies. Monoclonal antibodies may be prepared using hybridoma methods, such as those described by Kohler and Milstein, <u>supra</u>. In a hybridoma method, a mouse, hamster, or other appropriate host animal, is typically immunized (such as described above) with an immunizing agent to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the immunizing agent. Alternatively, the lymphocytes may be immunized <i>in vitro</i>.” ADE-33, P. 45, ll. 14-21.</p> <p>“The immunizing agent will typically include the Apo-2 polypeptide or a fusion protein thereof. An example of a suitable immunizing agent is an Apo-2-IgG fusion protein or chimeric molecule. A specific example of an Apo-2 ECD-IgG immunogen is described in Example 9 below.” ADE-33, P. 45, ll. 22-26.</p> <p>“The monoclonal antibodies may also be made by recombinant DNA methods, such as those described in U.S. Patent No. 4,816,567. DNA encoding the monoclonal antibodies of the invention can be readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies).” ADE-33, P. 46, ll. 37-42.</p> <p>“As described in the Examples below, anti-Apo-2 monoclonal antibodies have been prepared. One of these antibodies, 3F11.39.7, has been deposited with ATCC and has been assigned deposit accession no. HB-12456. In one embodiment, the monoclonal antibodies of the invention will have the same biological characteristics as the monoclonal antibodies secreted by the hybridoma cell line(s) deposited under Accession No. HB-12456. The term ‘biological characteristics’ is used to refer to the <i>in vitro</i> and/or <i>in vivo</i> activities or properties of the monoclonal antibody, such as the ability to specifically bind to Apo-2 or to substantially block, induce or enhance Apo-2 activation. As disclosed in the present specification, the 3F11.39.7 monoclonal antibody (HB-12456) is characterized as having agonistic activity for inducing apoptosis, binding to the Apo-2 receptor, having blocking activity as described in the</p>

Proposed Count	Support in Applicants' 60/074,119 Specification
	<p>Examples below, and having some cross-reactivity to DR4 but not to DcR1 or DcR2. Optionally, the monoclonal antibody will bind to the same epitope as the 3F11.39.7 antibody disclosed herein. This can be determined by conducting various assays, such as described herein and in the Examples. For instance, to determine whether a monoclonal antibody has the same specificity as the 3F11.39.7 antibody specifically disclosed, one can compare activity in Apo-2 blocking and apoptosis induction assays, such as those described in the Examples below.” <b>ADE-33, P. 47, ll. 20-41.</b></p> <p>“The antibodies of the invention may also comprise monovalent antibodies. Methods for preparing monovalent antibodies are well known in the art.” <b>ADE-33, P. 47, l. 42 - P. 48, l. 6.</b></p> <p>“The Apo-2 antibodies of the invention may further comprise humanized antibodies or human antibodies.” <b>ADE-33, P. 48, ll. 34-35.</b></p> <p>“Methods for humanizing non-human antibodies are well known in the art.” <b>ADE-33, P. 49, ll. 20-21.</b></p> <p>“The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important in order to reduce antigenicity. According to the ‘best-fit’ method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody [Sims et al., <i>J. Immunol.</i>, 151:2296 (1993); Chothia and Lesk, <i>J. Mol. Biol.</i>, 196:901 (1987)].” <b>ADE-33, P. 49, l. 36 - P. 50, l. 6.</b></p> <p>“It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three dimensional models of the parental and humanized sequences. Three dimensional immunoglobulin models are commonly</p>

Proposed Count	Support in Applicants' 60/074,119 Specification
	<p>available and are familiar to those skilled in the art.” ADE-33, P. 50, lns. 11-18.</p> <p>“Bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens. In the present case, one of the binding specificities is for the Apo-2, the other one is for any other antigen, and preferably for a cell-surface protein or receptor or receptor subunit.</p> <p>Methods for making bispecific antibodies are known in the art.” ADE-33, P. 51, ll. 7-14.</p> <p>“Other modifications of the Apo-2 antibodies are contemplated. For example, it may be desirable to modify the antibodies of the invention with respect to effector function, so as to enhance the therapeutic effectiveness of the antibodies.” ADE-33, P. 52, ll. 35-38.</p> <p>“The Apo-2 antibodies of the invention have therapeutic utility. Agonistic Apo-2 antibodies, for instance, may be employed to activate or stimulate apoptosis in cancer cells. The Apo-2 antibodies of the invention may also be useful in enhancing immune-mediated cell death in cells expressing Apo-2, for instance, through complement fixation or ADCC.” ADE-33, P. 53, ll. 16-21.</p> <p>“In a further embodiment of the invention, there are provided articles of manufacture and kits containing Apo-2 or Apo-2 antibodies which can be used, for instance, for the therapeutic or non-therapeutic applications described above.” ADE-33, P. 54, ll. 19-22.</p> <p>“Balb/c mice (obtained from Charles River Laboratories) were immunized by injecting 0.5µg/50µl of an Apo-2 ECD immunoadhesin protein (diluted in MPL-TDM adjuvant purchased from Ribi Immunochemical Research Inc., Hamilton, MT) 11 times into each hind foot pad at 3-4 day intervals. The Apo-2 ECD immunoadhesin protein was generated by fusing an extracellular domain sequence of Apo-2 (amino acids 1-184 shown in Fig. 1) to the hinge and Fc region of human immunoglobulin G<sub>1</sub> heavy chain in pRK5 as described previously [Ashkenazi et al., <u>Proc. Natl. Acad. Sci.</u>, 88:10535-10539 (1991)].” ADE-33, P. 64, ll. 14-22.</p>



Proposed Count	Support in Applicants' 60/074,119 Specification
	<p data-bbox="659 281 1438 537">“Of the hybridoma supernatants screened in the ELISA, 22 supernatants tested positive (calculated as approximately 4 times above background). The supernatants testing positive in the ELISA were further analyzed by FACS analysis using 9D cells (a human B lymphoid cell line expressing Apo-2; Genentech, Inc.) and FITC-conjugated goat anti-mouse IgG.” ADE-33, P. 65, ll. 29-34.</p> <p data-bbox="659 575 1438 646">“FACS analysis showed 8/22 supernatants were positive for anti-Apo-2 antibodies.” ADE-33, P. 66, ll. 7-9.</p> <p data-bbox="659 684 1438 865">“Figure 7 shows the FACS staining of 9D cells incubated with one of the Apo-2 antibodies, referred to as 3F11.39.7. As shown in FIG. 7, the 3F11.39.7 antibody recognizes the Apo-2 receptor expressed in 9D cells.” ADE-33, P. 66, ll. 10-13.</p> <p data-bbox="659 903 1438 1050">“Hybridoma supernatants and purified antibodies (as described in Example 9 above) were tested for activity to induce Apo-2 mediated 9D cell apoptosis.” ADE-33, P. 66, ll. 18-20.</p> <p data-bbox="659 1087 1438 1419">“As shown in Figure 8, the 3F11.39.7 antibody (in the absence of the goat anti-mouse IgG Fc) induced apoptosis in the 9D cells as compared to the control antibodies. Agonistic activity, however, was enhanced by Apo-2 receptor cross-linking in the presence of the goat anti-mouse IgG Fc (see Figure 9). This enhanced apoptosis (Figure 9) by the combination of antibodies is comparable to the apoptotic activity of Apo-2L in 9D cells (data not shown).” ADE-33, P. 66, ll. 33-39.</p> <p data-bbox="659 1457 1438 1528">“The ELISA was performed essentially as described in Example 9 above.</p> <p data-bbox="659 1533 1438 1675">The results are shown in Figure 11. The Apo-2 antibody 3F11.39.7 bound to Apo-2. The 3F11.39.7 antibody also showed some cross-reactivity to DR4, but not to DcR1 or DcR2.” P. 68, lns. 12-16.</p> <p data-bbox="659 1713 1438 1785">“3F11.39.7                      HB-12456                      January 13,1998” ADE-33, P. 69, l. 9.</p>

Proposed Count	Support in Applicants' 60/074,119 Specification
(a) binds to a soluble extracellular domain sequence of an Apo-2 polypeptide consisting of amino acids 54 to 182 of SEQ ID NO:1 and	<p data-bbox="656 247 1445 430">“In another embodiment, the invention provides an isolated extracellular domain (ECD) sequence of Apo-2. Optionally, the isolated extracellular domain sequence comprises amino acid residues 54 to 182 of Fig. 1 (SEQ ID NO:1).” ADE-33, P. 8, ll. 12-15.</p> <p data-bbox="656 472 1445 688">“FIG. 2A shows the derived amino acid sequence of a native sequence human Apo-2--the putative signal sequence is underlined, the putative transmembrane domain is boxed, and the putative death domain sequence is dash underlined. The cysteines of the two cysteine-rich domains are individually underlined.” ADE-33, P. 9, ll. 30-34.</p> <p data-bbox="656 730 1445 1056">“The ‘Apo-2 extracellular domain’ or ‘Apo-2 ECD’ refers to a form of Apo-2 which is essentially free of the transmembrane and cytoplasmic domains of Apo-2. Ordinarily, Apo-2 ECD will have less than 1% of such transmembrane and/or cytoplasmic domains and preferably, will have less than 0.5% of such domains. Optionally, Apo-2 ECD will comprise amino acid residues 54 to 182 of Fig. 1 (SEQ ID NO:1) or amino acid residues 1 to 182 of Fig. 1 (SEQ ID NO:1).” ADE-33, P. 11, l. 37 - P. 12, l. 5.</p> <p data-bbox="656 1098 1445 1570">“In general, the signal sequence may be a component of the vector, or it may be a part of the Apo-2 DNA that is inserted into the vector. The heterologous signal sequence selected preferably is one that is recognized and processed (i.e., cleaved by a signal peptidase) by the host cell...In mammalian cell expression the native Apo-2 presequence that normally directs insertion of Apo-2 in the cell membrane of human cells in vivo is satisfactory, although other mammalian signal sequences may be used to direct secretion of the protein, such as signal sequences from secreted polypeptides of the same or related species, as well as viral secretory leaders, for example, the herpes simplex glycoprotein D signal.” ADE-33, P. 19, ll. 18-37.</p> <p data-bbox="656 1612 1445 1858">“The entire nucleotide sequence of Apo-2 is shown in Figure 1 (SEQ ID NO:2). Clone 27868 (also referred to as pRK5-Apo-2 deposited as ATCC 209021, as indicated below) contains a single open reading frame with an apparent translational initiation site at nucleotide positions 140-142 [Kozak et al., supra] and ending at the stop codon found at nucleotide positions 1373-1375 (Fig. 1; SEQ ID</p>

Proposed Count	Support in Applicants' 60/074,119 Specification
	<p>NO:2). The predicted polypeptide precursor is 411 amino acids long, a type I transmembrane protein, and has a calculated molecular weight of approximately 45 kDa. Hydropathy analysis (not shown) suggested the presence of a signal sequence (residues 1-53), followed by an extracellular domain (residues 54-182), a transmembrane domain (residues 183-208), and an intracellular domain (residues 209-411) (Fig. 2A; SEQ ID NO:1). N-terminal amino acid sequence analysis of Apo-2-IgG expressed in 293 cells showed that the mature polypeptide starts at amino acid residue 54, indicating that the actual signal sequence comprises residues 1-53." <b>ADE-33, P. 57, ll. 24-38.</b></p> <p>"(The Apo-2 ECD construct included residues 183 and 184 shown in Figure. 1 to provide flexibility at the junction, even though residues 183 and 184 are predicted to be in the transmembrane region)." <b>ADE-33, P. 58, ll. 34-36.</b></p> <p>"A soluble Apo-2 ECD immunoadhesin construct was prepared. The Apo-2 ECD (amino acids 1-184 shown in Fig. 1) was fused to the hinge and Fc region of human immunoglobulin G<sub>1</sub> heavy chain in pRK5 as described previously [Ashkenazi et al., <u>Proc. Natl. Acad. Sci.</u>, <u>88</u>:10535-10539 (1991)]. The immunoadhesin was expressed by transient transfection into human 293 cells and purified from cell supernatants by protein A affinity chromatography, as described by Ashkenazi et al., <u>supra</u>." <b>ADE-33, P. 59, ll. 8-15.</b></p> <p>"Balb/c mice (obtained from Charles River Laboratories) were immunized by injecting 0.5 .mu.g/50 .mu.l of an Apo-2 ECD immunoadhesin protein (diluted in MPL-TDM adjuvant purchased from Ribi Immunochemical Research Inc., Hamilton, MT) 11 times into each hind foot pad at 3-4 day intervals. The Apo-2 ECD immunoadhesin protein was generated by fusing an extracellular domain sequence of Apo-2 (amino acids 1-184 shown in Fig. 1) to the hinge and Fc region of human immunoglobulin G<sub>1</sub> heavy chain in pRK5 as described previously [Ashkenazi et al., <u>Proc. Natl. Acad. Sci.</u>, <u>88</u>:10535-10539 (1991)]. The immunoadhesin protein was expressed by transient transfection into human 293 cells and purified from cell supernatants by protein A affinity chromatography, as described by Ashkenazi et al., <u>supra</u> (See also Example 2B above)." <b>ADE-33, P. 64, ll.</b></p>

Proposed Count	Support in Applicants' 60/074,119 Specification
<p>(b) induces apoptosis in at least one type of mammalian cancer cell <i>in vivo</i> or <i>ex vivo</i>.</p>	<p><b>14-25.</b></p> <p>“Figure 7 shows the FACS staining of 9D cells incubated with one of the Apo-2 antibodies, referred to as 3F11.39.7. As shown in Figure 7, the 3F11.39.7 antibody recognizes the Apo-2 receptor expressed in 9D cells.” <b>ADE-33, P. 66, ll. 10-13.</b></p> <p>“The results are shown in Figure 11. The Apo-2 antibody 3F11.39.7 bound to Apo-2. The 3F11.39.7 antibody also showed some cross-reactivity to DR4, but not to DcR1 or DcR2.” <b>ADE-33, P. 68, ll. 14-16.</b></p> <p>“The Apo-2 antibodies of the invention have therapeutic utility. Agonistic Apo-2 antibodies, for instance, may be employed to activate or stimulate apoptosis in cancer cells. The Apo-2 antibodies of the invention may also be useful in enhancing immune-mediated cell death in cells expressing Apo-2, for instance, through complement fixation or ADCC.” <b>ADE-33, P. 53, ll. 16-21.</b></p> <p>“As shown in Figure 8, the 3F11.39.7 antibody (in the absence of the goat anti-mouse IgG Fc) induced apoptosis in the 9D cells as compared to the control antibodies. Agonistic activity, however, was enhanced by Apo-2 receptor cross-linking in the presence of the goat anti-mouse IgG Fc (see Figure 9). This enhanced apoptosis (Figure 9) by the combination of antibodies is comparable to the apoptotic activity of Apo-2L in 9D cells (data not shown).” <b>ADE-33, P. 66, ll. 33-39.</b></p>

## APPENDIX H

### Constructive Reduction to Practice of Proposed Count in 09/079,029 Specification

Proposed Count	Support in Applicants' 09/079,029 Specification
65. A method of inducing apoptosis in mammalian cancer cells comprising	<p>“‘Biologically active’ and ‘desired biological activity’ for the purposes herein means (1) having the ability to modulate apoptosis (either in an agonistic or stimulating manner or in an antagonistic or blocking manner) in at least one type of mammalian cell <i>in vivo</i> or <i>ex vivo</i>; (2) having the ability to bind Apo-2 ligand; or (3) having the ability to modulate Apo-2 ligand signaling and Apo-2 ligand activity.” ADE-34, P. 18, ll. 18-24.</p> <p>“The terms ‘apoptosis’ and ‘apoptotic activity’ are used in a broad sense and refer to the orderly or controlled form of cell death in mammals that is typically accompanied by one or more characteristic cell changes, including condensation of cytoplasm, loss of plasma membrane microvilli, segmentation of the nucleus, degradation of chromosomal DNA or loss of mitochondrial function. This activity can be determined and measured, for instance, by cell viability assays, FACS analysis or DNA electrophoresis, all of which are known in the art.” ADE-34, P. 18, ll. 25-33.</p> <p>“The terms ‘cancer’ and ‘cancerous’ refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, blastoma, gastrointestinal cancer, renal cancer, pancreatic cancer, glioblastoma, neuroblastoma, cervical cancer, ovarian cancer, liver cancer, stomach cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer.” ADE-34, P. 18, l. 37 to P. 19, l. 10.</p> <p>“The term ‘mammal’ as used herein refers to any mammal classified as a mammal, including humans, cows, horses, dogs and cats. In a preferred embodiment of the invention, the mammal is a human.” ADE-34, P. 19, ll. 11-14.</p> <p>“Apo-2, as disclosed in the present specification, can be employed therapeutically to induce apoptosis in mammalian</p>

Proposed Count	Support in Applicants' 09/079,029 Specification
<p>exposing mammalian cancer cells to an effective amount of an Apo-2 agonist monoclonal antibody which</p>	<p>cells. This therapy can be accomplished for instance, using <i>in vivo</i> or <i>ex vivo</i> gene therapy techniques and includes the use of the death domain sequences disclosed herein. The Apo-2 chimeric molecules (including the chimeric molecules containing an extracellular domain sequence of Apo-2) comprising immunoglobulin sequences can also be employed therapeutically to inhibit apoptosis or NF-<math>\kappa</math>B induction by Apo-2L or by another ligand that Apo-2 binds to." <b>ADE-34, P. 45, ll. 8-16.</b></p> <p>"The Apo-2 antibodies of the invention have therapeutic utility. Agonistic Apo-2 antibodies, for instance, may be employed to activate or stimulate apoptosis in cancer cells. Accordingly, the invention provides methods for treating cancer using such Apo-2 antibodies." <b>ADE-34, P. 59, ll. 2-6.</b></p> <p>"Following administration of agonist to the mammal, the mammal's cancer and physiological condition can be monitored in various ways well known to the skilled practitioner. For instance, tumor mass may be observed physically or by standard x-ray imaging techniques." <b>ADE-34, P. 60, ll. 27-31.</b></p> <p>"In another embodiment, the invention provides an antibody which specifically binds to Apo-2. The antibody may be an agonistic, antagonistic or neutralizing antibody. Single-chain antibodies and dimeric molecules, in particular homodimeric molecules, comprising Apo-2 antibody are also provided." <b>ADE-34, P. 9, ll. 35-39.</b></p> <p>"A further embodiment of the invention provides articles of manufacture and kits that include Apo-2 or Apo-2 antibodies." <b>ADE-34, P. 10, ll. 4-5.</b></p> <p>"Figure 7 shows the FACS analysis of an Apo-2 antibody, 3F11.39.7 (illustrated by the bold lines) as compared to IgG controls (dotted lines). The 3F11.39.7 antibody recognized the Apo-2 receptor expressed in human 9D cells.</p> <p>Figure 8 is a graph showing percent (%) apoptosis induced in 9D cells by Apo-2 antibody 3F11.39.7, in the absence of goat anti-mouse IgG Fc.</p> <p>Figure 9 is a bar diagram showing percent (%) apoptosis,</p>

Proposed Count	Support in Applicants' 09/079,029 Specification
	<p>as compared to Apo-2L, in 9D cells by Apo-2 antibody 3F11.39.7 in the presence or absence of goat anti-mouse IgG Fc.” ADE-34, P. 11, ll. 13-22.</p> <p>“Figure 11 is a graph showing results of an ELISA testing binding of Apo-2 antibody 3F11.39.7 to Apo-2 and to other known Apo-2L receptors referred to as DR4, DcR1, and DcR2.</p> <p>Figure 12A is a graph showing the results of an ELISA assay evaluating binding of the 16E2 antibody to Apo-2, DR4, DcR1, DcR2 and CD4-Ig.</p> <p>Figure 12B is a graph showing the results of an ELISA assay evaluating binding of the 20E6 antibody to Apo-2, DR4, DcR1, DcR2 and CD4-Ig.</p> <p>Figure 12C is a graph showing the results of an ELISA assay evaluating binding of the 24C4 antibody to Apo-2, DR4, DcR1, DcR2 and CD4-Ig.</p> <p>Figure 13A is a graph showing agonistic activity of the 16E2 antibody, as compared to Apo-2L, in an apoptosis assay (crystal violet stain) using SK-MES-1 cells.</p> <p>Figure 13B is a bar diagram showing agonistic activity of the 16E2 antibody, as compared to 7D5 scFv antibody (an anti-tissue factor antibody), in an apoptosis assay (crystal violet stain) using SK-MES-1 cells.</p> <p>Figure 13C is a bar diagram showing agonistic activity of the 16E2 antibody, as compared to 7D5 scFv antibody, in an apoptosis assay (annexin V-biotin/streptavidin-[S<sup>35</sup>]) using SK-MES-1 cells.</p> <p>Figure 14A is a graph showing agonistic activity of the 20E6 antibody, as compared to Apo-2L, in an apoptosis assay (crystal violet stain) using SK-MES-1 cells.</p> <p>Figure 14B is a graph showing agonistic activity of the 20E6 antibody by a comparison between results obtained in the crystal violet and annexin V-biotin/streptavidin-[S<sup>35</sup>] apoptosis assays.</p> <p>Figure 14C is a graph showing agonistic activity of gD-tagged 16E2 antibody, as compared to Apo-2L, in an apoptosis assay (crystal violet stain) using SK-MES-1 cells.” ADE-34, P. 11, l. 26 to P. 12, l. 21.</p> <p>“The term ‘antibody’ is used in the broadest sense and specifically covers anti-Apo-2 monoclonal antibodies (including agonist, antagonist, and blocking or neutralizing antibodies) and anti-Apo-2 antibody compositions with</p>

Proposed Count	Support in Applicants' 09/079,029 Specification
	<p>polyepitopic specificity.</p> <p>The term 'monoclonal antibody' as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, <i>i.e.</i>, the individual antibodies comprising the population are identical except for possible naturally-occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen.</p> <p>The monoclonal antibodies herein include hybrid and recombinant antibodies produced by splicing a variable (including hypervariable) domain of an anti-Apo-2 antibody with a constant domain, or a light chain with a heavy chain, or a chain from one species with a chain from another species, or fusions with heterologous proteins, regardless of species of origin or immunoglobulin class or subclass designation, as well as antibody fragments (<i>e.g.</i>, Fab, F(ab')<sub>2</sub>, and Fv), so long as they exhibit the desired biological activity. See, <i>e.g.</i> U.S. Pat. No. 4,816,567 and Mage et al., in <u>Monoclonal Antibody Production Techniques and Applications</u>, pp.79-97 (Marcel Dekker, Inc.: New York, 1987).</p> <p>Thus, the modifier 'monoclonal' indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler and Milstein, <u>Nature</u>, 256:495 (1975), or may be made by recombinant DNA methods such as described in U.S. Pat. No. 4,816,567. The 'monoclonal antibodies' may also be isolated from phage libraries generated using the techniques described in McCafferty et al., <u>Nature</u>, 348:552-554 (1990), for example.</p> <p>'Single-chain Fv' or 'scFv' antibody fragments comprise the V<sub>H</sub> and V<sub>L</sub> domains of antibody, wherein these domains are present in a single polypeptide chain. Generally, the Fv polypeptide further comprises a polypeptide linker between the V<sub>H</sub> and V<sub>L</sub> domains which enables the scFv to form the desired structure for antigen binding. For a review of scFv</p>



Proposed Count	Support in Applicants' 09/079,029 Specification
	<p>see, e.g., Pluckthun, <u>The Pharmacology of Monoclonal Antibodies</u>, vol. 113, Rosenberg and Moore eds. Springer-Verlag, New York, pp. 269-315 (1994). The scFv antibody fragments of the present invention include but are not limited to the 16E2, 20E6 and 24C4 antibodies described in detail below. Within the scope of the scFv antibodies of the invention are scFv antibodies comprising VH and VL domains that include one or more of the CDR regions identified for the 16E2, 20E6 and 24C4 antibodies.</p> <p>'Humanized' forms of non-human (e.g. murine) antibodies are specific chimeric immunoglobulins, immunoglobulin chains, or fragments thereof (such as Fv, Fab, Fab', F(ab')<sub>2</sub> or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat, or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, the humanized antibody may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region or domain (Fc), typically that of a human immunoglobulin." ADE-34, P. 16, l. 21 - P. 18, l. 17.</p> <p>"The present invention provides newly identified and isolated Apo-2 polypeptides and Apo-2 antibodies. In particular, Applicants have identified and isolated various human Apo-2 polypeptides. The properties and characteristics of some of these Apo-2 polypeptides and anti-Apo-2 antibodies are described in further detail in the</p>

Proposed Count	Support in Applicants' 09/079,029 Specification
	<p>Examples below.” ADE-34, P. 19, l. 17-22.</p> <p>“A description follows as to how Apo-2, as well as Apo-2 chimeric molecules and anti-Apo-2 antibodies, may be prepared.” ADE-34, P. 19, ll. 26-27.</p> <p>“Preferably, the Ig is a human immunoglobulin when the chimeric molecule is intended for <i>in vivo</i> therapy for humans.” ADE-34, P. 44, ll. 21-22.</p> <p>“Apo-2 preparations are also useful in generating antibodies...” ADE-34, P. 45, ll. 28-29.</p> <p>“Modified forms of the Apo-2, such as the Apo-2-IgG chimeric molecules (immunoadhesins) described above, can be used as immunogens in producing anti-Apo-2 antibodies.” ADE-34, P. 45, ll. 35-37.</p> <p>“The present invention further provides anti-Apo-2 antibodies. Antibodies against Apo-2 may be prepared as follows. Exemplary antibodies include polyclonal, monoclonal, humanized, bispecific, and heteroconjugate antibodies.” ADE-34, P. 47, ll. 25-28.</p> <p>“The Apo-2 antibodies may, alternatively, be monoclonal antibodies. Monoclonal antibodies may be prepared using hybridoma methods, such as those described by Kohler and Milstein, <u>supra</u>. In a hybridoma method, a mouse, hamster, or other appropriate host animal, is typically immunized (such as described above) with an immunizing agent to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the immunizing agent. Alternatively, the lymphocytes may be immunized <i>in vitro</i>.” ADE-34, P. 48, ll. 16-24.</p> <p>“The immunizing agent will typically include the Apo-2 polypeptide or a fusion protein thereof. An example of a suitable immunizing agent is an Apo-2-IgG fusion protein or chimeric molecule. A specific example of an Apo-2 ECD-IgG immunogen is described in Example 9 below.” ADE-34, P. 48, ll. 25-29.</p> <p>“The monoclonal antibodies may also be made by recombinant DNA methods, such as those described in U.S.</p>

Proposed Count	Support in Applicants' 09/079,029 Specification
	<p>Patent No. 4,816,567. DNA encoding the monoclonal antibodies of the invention can be readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies).” <b>ADE-34, P. 50, ll. 3-9.</b></p> <p>“As described in the Examples below, anti-Apo-2 monoclonal antibodies have been prepared. One of these antibodies, 3F11.39.7, has been deposited with ATCC and has been assigned deposit accession no. HB-12456. In one embodiment, the monoclonal antibodies of the invention will have the same biological characteristics as the monoclonal antibodies secreted by the hybridoma cell line(s) deposited under Accession No. HB-12456. The term ‘biological characteristics’ is used to refer to the <i>in vitro</i> and/or <i>in vivo</i> activities or properties of the monoclonal antibody, such as the ability to specifically bind to Apo-2 or to substantially block, induce or enhance Apo-2 activation. As disclosed in the present specification, the 3F11.39.7 monoclonal antibody (HB-12456) is characterized as having agonistic activity for inducing apoptosis, binding to the Apo-2 receptor, having blocking activity as described in the Examples below, and having some cross-reactivity to DR4 but not to DcR1 or DcR2. Optionally, the monoclonal antibody will bind to the same epitope as the 3F11.39.7 antibody disclosed herein. This can be determined by conducting various assays, such as described herein and in the Examples. For instance, to determine whether a monoclonal antibody has the same specificity as the 3F11.39.7 antibody specifically disclosed, one can compare activity in Apo-2 blocking and apoptosis induction assays, such as those described in the Examples below.” <b>ADE-34, P. 50, l. 25 - P. 51, l. 8.</b></p> <p>“The antibodies of the invention may also comprise monovalent antibodies. Methods for preparing monovalent antibodies are well known in the art.” <b>ADE-34, P. 51, ll. 9-11.</b></p> <p>“The Apo-2 antibodies of the invention may further comprise humanized antibodies or human antibodies.” <b>ADE-34, P. 52, ll. 2-3.</b></p>

Proposed Count	Support in Applicants' 09/079,029 Specification
	<p data-bbox="659 243 1438 317">“Methods for humanizing non-human antibodies are well known in the art.” ADE-34, P. 52, ll. 28-29.</p> <p data-bbox="659 359 1438 758">“The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important in order to reduce antigenicity. According to the ‘best-fit’ method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody [Sims et al., J. Immunol., 151:2296 (1993); Chothia and Lesk, J. Mol. Biol., 196:901 (1987)].” ADE-34, P.53, ll. 7-15.</p> <p data-bbox="659 800 1438 1157">“It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three dimensional models of the parental and humanized sequences. Three dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art.” ADE-34, P. 53, ll. 21-28.</p> <p data-bbox="659 1199 1438 1860">“As described in the Examples below, anti-Apo-2 single-chain Fv (scFv) antibodies have been identified using a phage display library. Three of these antibodies, referred to herein as 16E2, 24C4 and 20E6, have been sequenced and characterized. The respective DNA and amino acid sequences and complementarity determining regions of these antibodies are shown in Figures 15A-15C and 16. In one embodiment of the invention, scFv Apo-2 antibodies will have the same biological characteristics as the 16E2, 24C4 or 20E6 antibodies identified herein. The term ‘biological characteristics’ is used to refer to the <i>in vitro</i> and/or <i>in vivo</i> activities or properties of the scFv antibody, such as the ability to specifically bind to Apo-2 or to substantially induce or enhance Apo-2 activation. As disclosed in the present specification, the 16E2, 24C4 and 20E6 antibodies are characterized as binding to Apo-2, having agonistic activity for inducing apoptosis, and having no cross-reactivity to DR4 or several of the other known</p>

Proposed Count	Support in Applicants' 09/079,029 Specification
	<p>molecules recognized by the Apo-2 ligand. Optionally, the scFv Apo-2 antibody will bind to the same epitope or epitopes recognized by the 16E2, 24C4 or 20E6 antibodies disclosed herein. This can be determined by conducting various assays, such as described herein and in the Examples. For instance, to determine whether a scFv antibody has the same specificity as the 16E2, 24C4 or 20E6 antibodies specifically disclosed, one can compare activity in apoptosis induction assays, such as those described in the Examples below.” <b>ADE-34, P. 54, l. 31 - P. 55, l. 16.</b></p> <p>“Optionally the scFv antibodies to Apo-2 may include antibodies which contain a VH and VL chain that include one or more complementarity determining region (CDR) amino acid sequences identified in Figure 16 for the 16E2, 20E6, or 24C4 antibodies.” <b>ADE-34, P. 55, ll. 17-20.</b></p> <p>“Bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens. In the present case, one of the binding specificities is for the Apo-2, the other one is for any other antigen, and preferably for a cell-surface protein or receptor or receptor subunit.</p> <p>Methods for making bispecific antibodies are known in the art.” <b>ADE-34, P. 55, ll. 23-30.</b></p> <p>“Other modifications of the Apo-2 antibodies are contemplated. For example, it may be desirable to modify the antibodies of the invention with respect to effector function, so as to enhance the therapeutic effectiveness of the antibodies.” <b>ADE-34, P. 57, ll. 16-19.</b></p> <p>“The agonist is preferably administered to the mammal in a carrier. Suitable carriers and their formulations are described in <u>Remington's Pharmaceutical Sciences</u>, 16th ed., 1980, Mack Publishing Co., edited by Oslo et al. Typically, an appropriate amount of a pharmaceutically-acceptable salt is used in the formulation to render the formulation isotonic. Examples of a pharmaceutically-acceptable carrier include saline, Ringer's solution and dextrose solution. The pH of the solution is preferably from about 5 to about 8, and more preferably from about 7 to about 7.5. Further carriers include sustained release</p>

Proposed Count	Support in Applicants' 09/079,029 Specification
	<p>preparations such as semipermeable matrices of solid hydrophobic polymers containing the agonist, which matrices are in the form of shaped articles, e.g., films, liposomes or microparticles. It will be apparent to those persons skilled in the art that certain carriers may be more preferable depending upon, for instance, the route of administration and concentration of agonist being administered." ADE-34, P. 59, ll. 9-25.</p> <p>"The agonist antibody can be administered to the mammal by injection (e.g., intravenous, intraperitoneal, subcutaneous, intramuscular), or by other methods such as infusion that ensure its delivery to the bloodstream in an effective form. The agonist may also be administered by intratumoral, peritumoral, intralesional, or perilesional routes, to exert local as well as systemic therapeutic effects. Local or intravenous injection is preferred." ADE-34, P. 59, ll. 26-33.</p> <p>"Effective dosages and schedules for administering the agonist antibody may be determined empirically, and making such determinations is within the skill in the art. Those skilled in the art will understand that the dosage of agonist that must be administered will vary depending on, for example, the mammal which will receive the agonist, the route of administration, the particular type of agonist used and other drugs being administered to the mammal. Guidance in selecting appropriate doses for antibody agonists is found in the literature on therapeutic uses of antibodies, e.g., <u>Handbook of Monoclonal Antibodies</u>, Ferrone et al., eds., Noyes Publications, Park Ridge, N.J., (1985) ch. 22 and pp. 303-357; Smith et al., <u>Antibodies in Human Diagnosis and Therapy</u>, Haber et al., eds., Raven Press, New York (1977) pp. 365-389. A typical daily dosage of the agonist used alone might range from about 1 µg/kg to up to 100 mg/kg of body weight or more per day, depending on the factors mentioned above." ADE-34, P. 59, l. 34 - P. 60, l. 10.</p> <p>"The agonist antibody may also be administered to the mammal in combination with effective amounts of one or more other therapeutic agents or in conjunction with radiation treatment. Therapeutic agents contemplated include chemotherapeutics as well as immunoadjuvants and</p>

Proposed Count	Support in Applicants' 09/079,029 Specification
	<p>cytokines. Chemotherapies contemplated by the invention include chemical substances or drugs which are known in the art and are commercially available, such as Doxorubicin, 5-Fluorouracil, Cytosine arabinoside ("Ara-C"), Cyclophosphamide, Thiotepa, Busulfan, Cytosin, Taxol, Methotrexate, Cisplatin, Melphalan, Vinblastine and Carboplatin. The agonist may be administered sequentially or concurrently with the one or more other therapeutic agents. The amounts of agonist and therapeutic agent depend, for example, on what type of drugs are used, the cancer being treated, and the scheduling and routes of administration but would generally be less than if each were used individually." ADE-34, P. 60, ll. 11-26.</p> <p>"Following administration of agonist to the mammal, the mammal's cancer and physiological condition can be monitored in various ways well known to the skilled practitioner. For instance, tumor mass may be observed physically or by standard x-ray imaging techniques." ADE-34, P. 60, ll. 27-31.</p> <p>"The Apo-2 antibodies of the invention may also be useful in enhancing immune-mediated cell death in cells expressing Apo-2, for instance, through complement fixation or ADCC." ADE-34, P. 60, ll. 32-34.</p> <p>"In a further embodiment of the invention, there are provided articles of manufacture and kits containing Apo-2 or Apo-2 antibodies which can be used, for instance, for the therapeutic or non-therapeutic applications described above." ADE-34, P. 61, l. 37 - P. 62, l. 1.</p> <p>"Balb/c mice (obtained from Charles River Laboratories) were immunized by injecting 0.5µg/50µl of an Apo-2 ECD immunoadhesin protein (diluted in MPL-TDM adjuvant purchased from Ribi Immunochemical Research Inc., Hamilton, MT) 11 times into each hind foot pad at 3-4 day intervals. The Apo-2 ECD immunoadhesin protein was generated by fusing an extracellular domain sequence of Apo-2 (amino acids 1-184 shown in Fig. 1) to the hinge and Fc region of human immunoglobulin G<sub>1</sub> heavy chain in pRK5 as described previously [Ashkenazi et al., <u>Proc. Natl. Acad. Sci.</u>, 88:10535-10539 (1991)]." ADE-34, P. 74, ll. 4-12.</p>

Proposed Count	Support in Applicants' 09/079,029 Specification
	<p data-bbox="657 279 1437 533">“Of the hybridoma supernatants screened in the ELISA, 22 supernatants tested positive (calculated as approximately 4 times above background). The supernatants testing positive in the ELISA were further analyzed by FACS analysis using 9D cells (a human B lymphoid cell line expressing Apo-2; Genentech, Inc.) and FITC-conjugated goat anti-mouse IgG.” ADE-34, P. 75, ll. 22-27.</p> <p data-bbox="657 573 1437 646">“FACS analysis showed 8/22 supernatants were positive for anti-Apo-2 antibodies.” ADE-34, P. 75, ll. 36-37.</p> <p data-bbox="657 686 1437 865">“Figure 7 shows the FACS staining of 9D cells incubated with one of the Apo-2 antibodies, referred to as 3F11.39.7. As shown in Figure 7, the 3F11.39.7 antibody recognizes the Apo-2 receptor expressed in 9D cells.” ADE-34, P. 76, ll. 1-4.</p> <p data-bbox="657 905 1437 1045">“Hybridoma supernatants and purified antibodies (as described in Example 9 above) were tested for activity to induce Apo-2 mediated 9D cell apoptosis.” ADE-34, P. 76, ll. 11-13.</p> <p data-bbox="657 1085 1437 1413">“As shown in Figure 8, the 3F11.39.7 antibody (in the absence of the goat anti-mouse IgG Fc) induced apoptosis in the 9D cells as compared to the control antibodies. Agonistic activity, however, was enhanced by Apo-2 receptor cross-linking in the presence of the goat anti-mouse IgG Fc (see Figure 9). This enhanced apoptosis (Figure 9) by the combination of antibodies is comparable to the apoptotic activity of Apo-2L in 9D cells (data not shown).” ADE-34, P. 76, ll. 28-35.</p> <p data-bbox="657 1453 1437 1596">“The results are shown in FIG. 11. The Apo-2 antibody 3F11.39.7 bound to Apo-2. The 3F11.39.7 antibody also showed some cross-reactivity to DR4, but not to DcR1 or DcR2.” ADE-34, P. 78, ll. 12-14.</p> <p data-bbox="657 1635 1437 1745">“Purified scFv antibodies (as described in Example 14 above) were tested for ability to induce Apo-2 mediated apoptosis.” ADE-34, P. 85, ll. 13-14.</p> <p data-bbox="657 1785 1437 1854">“As shown in Figures 13C and 14B, the 16E2 and 20E6 antibodies agonistically induced apoptosis in SK-MES-1</p>



Proposed Count	Support in Applicants' 09/079,029 Specification
(a) binds to a soluble extracellular domain sequence of an Apo-2 polypeptide consisting of amino acids 54 to 182 of SEQ ID NO:1 and	cells." ADE-34, P. 86, ll. 13-14.
	<p>"In addition to the annexin V-biotin/streptavidin-[S-35] assay described above, scFv antibodies (as described in Example 14 above) were tested for activity to induce Apo-2 mediated apoptosis via assays utilizing crystal violet." ADE-34, P. 86, ll. 17-20.</p>
	<p>"As shown in Figures 13A, 13B, 14A and 14B, the 16E2 and 20E6 antibodies agonistically induced apoptosis in SK-MES-1 cells." ADE-34, P. 87, ll. 15-16.</p>
	<p>"A purified gD-tagged form of 16E2 scFv was tested for ability to induce Apo-2 mediated apoptosis in a crystal violet assay as described in Example 15 above." ADE-34, P. 87, ll. 22-24.</p>
	<p>"The apoptosis assay was performed essentially as described in Example 15(B) above except that samples were serially diluted 1:3 in the plates and the 16E2-gD tagged antibody was tested in addition to two other preparations of 16E2 scFv (referred to as Prep. A and Prep. B in Figure 14C). The results of the assay showing apoptosis induction in SK-MES-1 cells by 16E2-gD antibody are illustrated in Figure 14C." ADE-34, P. 88, ll. 30-36.</p> <p>"3F11.39.7 HB-12456 January 13, 1998" ADE-34, P. 89, ll. 8-9.</p>
	<p>"In another embodiment, the invention provides an isolated extracellular domain (ECD) sequence of Apo-2. Optionally, the isolated extracellular domain sequence comprises amino acid residues 54 to 182 of Fig. 1 (SEQ ID NO:1)." ADE-34, P. 8, ll. 25-28.</p> <p>"FIG. 2A shows the derived amino acid sequence of a native sequence human Apo-2--the putative signal sequence is underlined, the putative transmembrane domain is boxed, and the putative death domain sequence is dash underlined. The cysteines of the two cysteine-rich domains are individually underlined." ADE-34, P. 10, ll. 10-14.</p> <p>"The 'Apo-2 extracellular domain' or 'Apo-2 ECD' refers</p>

Proposed Count	Support in Applicants' 09/079,029 Specification
	<p>to a form of Apo-2 which is essentially free of the transmembrane and cytoplasmic domains of Apo-2. Ordinarily, Apo-2 ECD will have less than 1% of such transmembrane and/or cytoplasmic domains and preferably, will have less than 0.5% of such domains. Optionally, Apo-2 ECD will comprise amino acid residues 54 to 182 of Fig. 1 (SEQ ID NO:1) or amino acid residues 1 to 182 of Fig. 1 (SEQ ID NO:1)." <b>ADE-34, P. 13, ll. 30-37.</b></p> <p>"In general, the signal sequence may be a component of the vector, or it may be a part of the Apo-2 DNA that is inserted into the vector. The heterologous signal sequence selected preferably is one that is recognized and processed (i.e., cleaved by a signal peptidase) by the host cell...In mammalian cell expression the native Apo-2 presequence that normally directs insertion of Apo-2 in the cell membrane of human cells in vivo is satisfactory, although other mammalian signal sequences may be used to direct secretion of the protein, such as signal sequences from secreted polypeptides of the same or related species, as well as viral secretory leaders, for example, the herpes simplex glycoprotein D signal." <b>ADE-34, P. 22, l. 29 - P. 23, l. 10.</b></p> <p>"The entire nucleotide sequence of Apo-2 is shown in Figure 1 (SEQ ID NO:2). Clone 27868 (also referred to as pRK5-Apo-2 deposited as ATCC 209021, as indicated below) contains a single open reading frame with an apparent translational initiation site at nucleotide positions 140-142 [Kozak et al., <i>supra</i>] and ending at the stop codon found at nucleotide positions 1373-1375 (Fig. 1; SEQ ID NO:2). The predicted polypeptide precursor is 411 amino acids long, a type I transmembrane protein, and has a calculated molecular weight of approximately 45 kDa. Hydropathy analysis (not shown) suggested the presence of a signal sequence (residues 1-53), followed by an extracellular domain (residues 54-182), a transmembrane domain (residues 183-208), and an intracellular domain (residues 209-411) (Fig. 2A; SEQ ID NO:1). N-terminal amino acid sequence analysis of Apo-2-IgG expressed in 293 cells showed that the mature polypeptide starts at amino acid residue 54, indicating that the actual signal sequence comprises residues 1-53." <b>ADE-34, P. 65, ll. 9-24.</b></p> <p>"(The Apo-2 ECD construct included residues 183 and 184</p>

Proposed Count	Support in Applicants' 09/079,029 Specification
	<p>shown in Figure. 1 to provide flexibility at the junction, even though residues 183 and 184 are predicted to be in the transmembrane region)." ADE-34, P. 66, ll. 20-23.</p> <p>"A soluble Apo-2 ECD immunoadhesin construct was prepared. The Apo-2 ECD (amino acids 1-184 shown in Fig. 1) was fused to the hinge and Fc region of human immunoglobulin G<sub>1</sub> heavy chain in pRK5 as described previously [Ashkenazi et al., <u>Proc. Natl. Acad. Sci.</u>, 88:10535-10539 (1991)]. The immunoadhesin was expressed by transient transfection into human 293 cells and purified from cell supernatants by protein A affinity chromatography, as described by Ashkenazi et al., <u>supra</u>." ADE-34, P. 66, l. 33 - P. 67, l. 2.</p> <p>"Balb/c mice (obtained from Charles River Laboratories) were immunized by injecting 0.5 .mu.g/50 .mu.l of an Apo-2 ECD immunoadhesin protein (diluted in MPL-TDM adjuvant purchased from Ribi Immunochemical Research Inc., Hamilton, MT) 11 times into each hind foot pad at 3-4 day intervals. The Apo-2 ECD immunoadhesin protein was generated by fusing an extracellular domain sequence of Apo-2 (amino acids 1-184 shown in Fig. 1) to the hinge and Fc region of human immunoglobulin G<sub>1</sub> heavy chain in pRK5 as described previously [Ashkenazi et al., <u>Proc. Natl. Acad. Sci.</u>, 88:10535-10539 (1991)]. The immunoadhesin protein was expressed by transient transfection into human 293 cells and purified from cell supernatants by protein A affinity chromatography, as described by Ashkenazi et al., <u>supra</u> (See also Example 2B above)." ADE-34, P. 74, ll. 4-16.</p> <p>"Figure 7 shows the FACS staining of 9D cells incubated with one of the Apo-2 antibodies, referred to as 3F11.39.7. As shown in Figure 7, the 3F11.39.7 antibody recognizes the Apo-2 receptor expressed in 9D cells." ADE-34, P. 76, ll. 1-4.</p> <p>"The results are shown in FIG. 11. The Apo-2 antibody 3F11.39.7 bound to Apo-2. The 3F11.39.7 antibody also showed some cross-reactivity to DR4, but not to DcR1 or DcR2." ADE-34, P. 78, ll. 12-14.</p> <p>"A phage library was selected using soluble biotinylated</p>

Proposed Count	Support in Applicants' 09/079,029 Specification
(b) induces apoptosis in at least one type of mammalian cancer cell <i>in vivo</i> or <i>ex vivo</i> .	antigen and streptavidin-coated paramagnetic beads. The antigen, an Apo-2 ECD immunoadhesin prepared as described in Example 2B above, was biotinylated using IMMUNOPURE NHS-biotin (biotiny-N-hydroxy-succinimide, Pierce) according to manufacturer's instructions." ADE-34, P. 79, ll. 5-10.
	"To evaluate the specificity of each of the scFv clones, ELISA assays were performed to evaluate binding of 16E2, 20E6 and 24C4 to Apo-2 ECD-Ig, DR4-Ig, DcR1-Ig, DcR2-Ig and CD4-Ig (described above and in Example 12)." ADE-34, P. 83, l. 35 - P. 84, l. 2.
	"As illustrated in Figures 12A, 12B and 12C, the ELISA assays showed that each of these antibodies exhibited a relatively high degree of specificity for Apo-2." P. 84, lns. 23-25.
	"The Apo-2 antibodies of the invention have therapeutic utility. Agonistic Apo-2 antibodies, for instance, may be employed to activate or stimulate apoptosis in cancer cells. Accordingly, the invention provides methods for treating cancer using such Apo-2 antibodies." ADE-34, P. 59, ll. 2-6.
	"As shown in Figure 8, the 3F11.39.7 antibody (in the absence of the goat anti-mouse IgG Fc) induced apoptosis in the 9D cells as compared to the control antibodies. Agonistic activity, however, was enhanced by Apo-2 receptor cross-linking in the presence of the goat anti-mouse IgG Fc (see Figure 9). This enhanced apoptosis (Figure 9) by the combination of antibodies is comparable to the apoptotic activity of Apo-2L in 9D cells (data not shown)." ADE-34, P. 76, ll. 28-35.
	"Purified scFv antibodies (as described in Example 14 above) were tested for ability to induce Apo-2 mediated apoptosis." ADE-34, P. 85, ll. 13-14.  "As shown in Figures 13C and 14B, the 16E2 and 20E6 antibodies agonistically induced apoptosis in SK-MES-1 cells." ADE-34, P. 86, ll. 13-14.

Proposed Count	Support in Applicants' 09/079,029 Specification
	<p data-bbox="659 243 1438 422">“In addition to the annexin V-biotin/streptavidin-[S-35] assay described above, scFv antibodies (as described in Example 14 above) were tested for activity to induce Apo-2 mediated apoptosis via assays utilizing crystal violet.” <b>ADE-34, P. 86, ll. 17-20.</b></p> <p data-bbox="659 464 1438 569">“As shown in Figures 13A, 13B, 14A and 14B, the 16E2 and 20E6 antibodies agonistically induced apoptosis in SK-MES-1 cells.” <b>ADE-34, P. 87, ll. 15-16.</b></p> <p data-bbox="659 611 1438 758">“A purified gD-tagged form of 16E2 scFv was tested for ability to induce Apo-2 mediated apoptosis in a crystal violet assay as described in Example 15 above.” <b>ADE-34, P. 87, ll. 22-24.</b></p> <p data-bbox="659 800 1438 1083">“The apoptosis assay was performed essentially as described in Example 15(B) above except that samples were serially diluted 1:3 in the plates and the 16E2-gD tagged antibody was tested in addition to two other preparations of 16E2 scFv (referred to as Prep. A and Prep. B in Figure 14C). The results of the assay showing apoptosis induction in SK-MES-1 cells by 16E2-gD antibody are illustrated in Figure 14C.” <b>ADE-34, P. 88, ll. 30-36.</b></p>

## **APPENDIX I**

### **Constructive Reduction to Practice of Proposed Count in 10/052,798 Specification**

<b>Proposed Count</b>	<b>Support in Applicants' 10/052,798 Specification</b>
65. A method of inducing apoptosis in mammalian cancer cells comprising	<p>“‘Biologically active’ and ‘desired biological activity’ for the purposes herein means (1) having the ability to modulate apoptosis (either in an agonistic or stimulating manner or in an antagonistic or blocking manner) in at least one type of mammalian cell <i>in vivo</i> or <i>ex vivo</i>; (2) having the ability to bind Apo-2 ligand; or (3) having the ability to modulate Apo-2 ligand signaling and Apo-2 ligand activity.” ADE-35, P. 18, ll. 18-24.</p> <p>“The terms ‘apoptosis’ and ‘apoptotic activity’ are used in a broad sense and refer to the orderly or controlled form of cell death in mammals that is typically accompanied by one or more characteristic cell changes, including condensation of cytoplasm, loss of plasma membrane microvilli, segmentation of the nucleus, degradation of chromosomal DNA or loss of mitochondrial function. This activity can be determined and measured, for instance, by cell viability assays, FACS analysis or DNA electrophoresis, all of which are known in the art.” ADE-35, P. 18, ll. 25-33.</p> <p>“The terms ‘cancer’ and ‘cancerous’ refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, blastoma, gastrointestinal cancer, renal cancer, pancreatic cancer, glioblastoma, neuroblastoma, cervical cancer, ovarian cancer, liver cancer, stomach cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer.” ADE-35, P. 18, l. 37 - P. 19, l. 10.</p> <p>“The term ‘mammal’ as used herein refers to any mammal classified as a mammal, including humans, cows, horses, dogs and cats. In a preferred embodiment of the invention, the mammal is a human.” ADE-35, P. 19, ll. 11-14.</p> <p>“Apo-2, as disclosed in the present specification, can be employed therapeutically to induce apoptosis in mammalian</p>

Proposed Count	Support in Applicants' 10/052,798 Specification
<p>exposing mammalian cancer cells to an effective amount of an Apo-2 agonist monoclonal antibody which</p>	<p>cells. This therapy can be accomplished for instance, using <i>in vivo</i> or <i>ex vivo</i> gene therapy techniques and includes the use of the death domain sequences disclosed herein. The Apo-2 chimeric molecules (including the chimeric molecules containing an extracellular domain sequence of Apo-2) comprising immunoglobulin sequences can also be employed therapeutically to inhibit apoptosis or NF-<math>\kappa</math>B induction by Apo-2L or by another ligand that Apo-2 binds to." ADE-35, P. 45, ll. 8-16.</p> <p>"The Apo-2 antibodies of the invention have therapeutic utility. Agonistic Apo-2 antibodies, for instance, may be employed to activate or stimulate apoptosis in cancer cells. Accordingly, the invention provides methods for treating cancer using such Apo-2 antibodies." ADE-35, P. 59, ll. 2-6.</p> <p>"Following administration of agonist to the mammal, the mammal's cancer and physiological condition can be monitored in various ways well known to the skilled practitioner. For instance, tumor mass may be observed physically or by standard x-ray imaging techniques." ADE-35, P. 60, ll. 27-31.</p> <p>"In another embodiment, the invention provides an antibody which specifically binds to Apo-2. The antibody may be an agonistic, antagonistic or neutralizing antibody. Single-chain antibodies and dimeric molecules, in particular homodimeric molecules, comprising Apo-2 antibody are also provided." ADE-35, P. 9, ll. 35-39.</p> <p>"A further embodiment of the invention provides articles of manufacture and kits that include Apo-2 or Apo-2 antibodies." ADE-35, P. 10, ll. 3-4.</p> <p>"Figure 7 shows the FACS analysis of an Apo-2 antibody, 3F11.39.7 (illustrated by the bold lines) as compared to IgG controls (dotted lines). The 3F11.39.7 antibody recognized the Apo-2 receptor expressed in human 9D cells.</p> <p>Figure 8 is a graph showing percent (%) apoptosis induced in 9D cells by Apo-2 antibody 3F11.39.7, in the absence of goat anti-mouse IgG Fc.</p> <p>Figure 9 is a bar diagram showing percent (%) apoptosis,</p>

Proposed Count	Support in Applicants' 10/052,798 Specification
	<p>as compared to Apo-2L, in 9D cells by Apo-2 antibody 3F11.39.7 in the presence or absence of goat anti-mouse IgG Fc.” ADE-35, P. 11, ll. 13-22.</p> <p>“Figure 11 is a graph showing results of an ELISA testing binding of Apo-2 antibody 3F11.39.7 to Apo-2 and to other known Apo-2L receptors referred to as DR4, DcR1, and DcR2.</p> <p>Figure 12A is a graph showing the results of an ELISA assay evaluating binding of the 16E2 antibody to Apo-2, DR4, DcR1, DcR2 and CD4-Ig.</p> <p>Figure 12B is a graph showing the results of an ELISA assay evaluating binding of the 20E6 antibody to Apo-2, DR4, DcR1, DcR2 and CD4-Ig.</p> <p>Figure 12C is a graph showing the results of an ELISA assay evaluating binding of the 24C4 antibody to Apo-2, DR4, DcR1, DcR2 and CD4-Ig.</p> <p>Figure 13A is a graph showing agonistic activity of the 16E2 antibody, as compared to Apo-2L, in an apoptosis assay (crystal violet stain) using SK-MES-1 cells.</p> <p>Figure 13B is a bar diagram showing agonistic activity of the 16E2 antibody, as compared to 7D5 scFv antibody (an anti-tissue factor antibody), in an apoptosis assay (crystal violet stain) using SK-MES-1 cells.</p> <p>Figure 13C is a bar diagram showing agonistic activity of the 16E2 antibody, as compared to 7D5 scFv antibody, in an apoptosis assay (annexin V-biotin/streptavidin-[S<sup>35</sup>]) using SK-MES-1 cells.</p> <p>Figure 14A is a graph showing agonistic activity of the 20E6 antibody, as compared to Apo-2L, in an apoptosis assay (crystal violet stain) using SK-MES-1 cells.</p> <p>Figure 14B is a graph showing agonistic activity of the 20E6 antibody by a comparison between results obtained in the crystal violet and annexin V-biotin/streptavidin-[S<sup>35</sup>] apoptosis assays.</p> <p>Figure 14C is a graph showing agonistic activity of gD-tagged 16E2 antibody, as compared to Apo-2L, in an apoptosis assay (crystal violet stain) using SK-MES-1 cells.” ADE-35, P. 11, l. 26 to P. 12, l. 21.</p> <p>“The term ‘antibody’ is used in the broadest sense and specifically covers anti-Apo-2 monoclonal antibodies (including agonist, antagonist, and blocking or neutralizing antibodies) and anti-Apo-2 antibody compositions with</p>



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	<p>polyepitopic specificity.</p> <p>The term 'monoclonal antibody' as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, <i>i.e.</i>, the individual antibodies comprising the population are identical except for possible naturally-occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen.</p> <p>The monoclonal antibodies herein include hybrid and recombinant antibodies produced by splicing a variable (including hypervariable) domain of an anti-Apo-2 antibody with a constant domain, or a light chain with a heavy chain, or a chain from one species with a chain from another species, or fusions with heterologous proteins, regardless of species of origin or immunoglobulin class or subclass designation, as well as antibody fragments (<i>e.g.</i>, Fab, F(ab')<sub>2</sub>, and Fv), so long as they exhibit the desired biological activity. See, <i>e.g.</i> U.S. Pat. No. 4,816,567 and Mage et al., in <u>Monoclonal Antibody Production Techniques and Applications</u>, pp.79-97 (Marcel Dekker, Inc.: New York, 1987).</p> <p>Thus, the modifier 'monoclonal' indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler and Milstein, <u>Nature</u>, 256:495 (1975), or may be made by recombinant DNA methods such as described in U.S. Pat. No. 4,816,567. The 'monoclonal antibodies' may also be isolated from phage libraries generated using the techniques described in McCafferty et al., <u>Nature</u>, 348:552-554 (1990), for example.</p> <p>'Single-chain Fv' or 'scFv' antibody fragments comprise the V<sub>H</sub> and V<sub>L</sub> domains of antibody, wherein these domains are present in a single polypeptide chain. Generally, the F<sub>V</sub> polypeptide further comprises a polypeptide linker between the V<sub>H</sub> and V<sub>L</sub> domains which enables the scFv to form the desired structure for antigen binding. For a review of scFv</p>

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	<p>see, e.g., Pluckthun, <u>The Pharmacology of Monoclonal Antibodies</u>, vol. 113, Rosenberg and Moore eds. Springer-Verlag, New York, pp. 269-315 (1994). The scFv antibody fragments of the present invention include but are not limited to the 16E2, 20E6 and 24C4 antibodies described in detail below. Within the scope of the scFv antibodies of the invention are scFv antibodies comprising VH and VL domains that include one or more of the CDR regions identified for the 16E2, 20E6 and 24C4 antibodies.</p> <p>'Humanized' forms of non-human (e.g. murine) antibodies are specific chimeric immunoglobulins, immunoglobulin chains, or fragments thereof (such as Fv, Fab, Fab', F(ab')<sub>2</sub> or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat, or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, the humanized antibody may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region or domain (Fc), typically that of a human immunoglobulin." ADE-35, P. 16, l. 21 - P. 18, l. 17.</p> <p>"The present invention provides newly identified and isolated Apo-2 polypeptides and Apo-2 antibodies. In particular, Applicants have identified and isolated various human Apo-2 polypeptides. The properties and characteristics of some of these Apo-2 polypeptides and anti-Apo-2 antibodies are described in further detail in the</p>

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	<p data-bbox="662 237 1242 279">Examples below." ADE-35, P. 19, ll. 17-22.</p> <p data-bbox="662 310 1409 426">"A description follows as to how Apo-2, as well as Apo-2 chimeric molecules and anti-Apo-2 antibodies, may be prepared." ADE-35, P. 19, ll. 26-27.</p> <p data-bbox="662 457 1398 573">"Preferably, the Ig is a human immunoglobulin when the chimeric molecule is intended for <i>in vivo</i> therapy for humans." ADE-35, P. 44, ll. 21-22.</p> <p data-bbox="662 604 1295 678">"Apo-2 preparations are also useful in generating antibodies..." ADE-35, P. 45, ll. 28-29.</p> <p data-bbox="662 709 1442 867">"Modified forms of the Apo-2, such as the Apo-2-IgG chimeric molecules (immunoadhesins) described above, can be used as immunogens in producing anti-Apo-2 antibodies." ADE-35, P. 45, ll. 35-37.</p> <p data-bbox="662 898 1398 1087">"The present invention further provides anti-Apo-2 antibodies. Antibodies against Apo-2 may be prepared as follows. Exemplary antibodies include polyclonal, monoclonal, humanized, bispecific, and heteroconjugate antibodies." ADE-35, P. 47, ll. 25-28.</p> <p data-bbox="662 1119 1433 1497">"The Apo-2 antibodies may, alternatively, be monoclonal antibodies. Monoclonal antibodies may be prepared using hybridoma methods, such as those described by Kohler and Milstein, <u>supra</u>. In a hybridoma method, a mouse, hamster, or other appropriate host animal, is typically immunized (such as described above) with an immunizing agent to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the immunizing agent. Alternatively, the lymphocytes may be immunized <i>in vitro</i>." ADE-35, P. 48, ll. 16-24.</p> <p data-bbox="662 1528 1409 1749">"The immunizing agent will typically include the Apo-2 polypeptide or a fusion protein thereof. An example of a suitable immunizing agent is an Apo-2-IgG fusion protein or chimeric molecule. A specific example of an Apo-2 ECD-IgG immunogen is described in Example 9 below." ADE-35, P. 48, ll. 25-29.</p> <p data-bbox="662 1780 1433 1854">"The monoclonal antibodies may also be made by recombinant DNA methods, such as those described in U.S.</p>

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	<p data-bbox="659 245 1430 464">Patent No. 4,816,567. DNA encoding the monoclonal antibodies of the invention can be readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies).” <b>ADE-35, P. 50, ll. 3-9.</b></p> <p data-bbox="659 504 1430 1493">“As described in the Examples below, anti-Apo-2 monoclonal antibodies have been prepared. One of these antibodies, 3F11.39.7, has been deposited with ATCC and has been assigned deposit accession no. HB-12456. In one embodiment, the monoclonal antibodies of the invention will have the same biological characteristics as the monoclonal antibodies secreted by the hybridoma cell line(s) deposited under Accession No. HB-12456. The term ‘biological characteristics’ is used to refer to the <i>in vitro</i> and/or <i>in vivo</i> activities or properties of the monoclonal antibody, such as the ability to specifically bind to Apo-2 or to substantially block, induce or enhance Apo-2 activation. As disclosed in the present specification, the 3F11.39.7 monoclonal antibody (HB-12456) is characterized as having agonistic activity for inducing apoptosis, binding to the Apo-2 receptor, having blocking activity as described in the Examples below, and having some cross-reactivity to DR4 but not to DcR1 or DcR2. Optionally, the monoclonal antibody will bind to the same epitope as the 3F11.39.7 antibody disclosed herein. This can be determined by conducting various assays, such as described herein and in the Examples. For instance, to determine whether a monoclonal antibody has the same specificity as the 3F11.39.7 antibody specifically disclosed, one can compare activity in Apo-2 blocking and apoptosis induction assays, such as those described in the Examples below.” <b>ADE-35, P. 50, l. 25 - P. 51, l. 8.</b></p> <p data-bbox="659 1533 1430 1675">“The antibodies of the invention may also comprise monovalent antibodies. Methods for preparing monovalent antibodies are well known in the art.” <b>ADE-35, P. 51, ll. 9-11.</b></p> <p data-bbox="659 1715 1430 1822">“The Apo-2 antibodies of the invention may further comprise humanized antibodies or human antibodies.” <b>ADE-35, P. 52, ll. 2-3.</b></p>

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	<p data-bbox="656 237 1445 310">“Methods for humanizing non-human antibodies are well known in the art.” ADE-35, P. 52, ll. 28-29.</p> <p data-bbox="656 352 1445 751">“The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important in order to reduce antigenicity. According to the ‘best-fit’ method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody [Sims et al., <i>J. Immunol.</i>, 151:2296 (1993); Chothia and Lesk, <i>J. Mol. Biol.</i>, 196:901 (1987)].” ADE-35, P.53, ll. 7-15.</p> <p data-bbox="656 793 1445 1161">“It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three dimensional models of the parental and humanized sequences. Three dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art.” ADE-35, P. 53, ll. 21-28.</p> <p data-bbox="656 1203 1445 1854">“As described in the Examples below, anti-Apo-2 single-chain Fv (scFv) antibodies have been identified using a phage display library. Three of these antibodies, referred to herein as 16E2, 24C4 and 20E6, have been sequenced and characterized. The respective DNA and amino acid sequences and complementarity determining regions of these antibodies are shown in Figures 15A-15C and 16. In one embodiment of the invention, scFv Apo-2 antibodies will have the same biological characteristics as the 16E2, 24C4 or 20E6 antibodies identified herein. The term ‘biological characteristics’ is used to refer to the <i>in vitro</i> and/or <i>in vivo</i> activities or properties of the scFv antibody, such as the ability to specifically bind to Apo-2 or to substantially induce or enhance Apo-2 activation. As disclosed in the present specification, the 16E2, 24C4 and 20E6 antibodies are characterized as binding to Apo-2, having agonistic activity for inducing apoptosis, and having no cross-reactivity to DR4 or several of the other known</p>

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	<p>molecules recognized by the Apo-2 ligand. Optionally, the scFv Apo-2 antibody will bind to the same epitope or epitopes recognized by the 16E2, 24C4 or 20E6 antibodies disclosed herein. This can be determined by conducting various assays, such as described herein and in the Examples. For instance, to determine whether a scFv antibody has the same specificity as the 16E2, 24C4 or 20E6 antibodies specifically disclosed, one can compare activity in apoptosis induction assays, such as those described in the Examples below.” <b>ADE-35, P. 54, l. 31 - P. 55, l. 16.</b></p> <p>“Optionally the scFv antibodies to Apo-2 may include antibodies which contain a VH and VL chain that include one or more complementarity determining region (CDR) amino acid sequences identified in Figure 16 for the 16E2, 20E6, or 24C4 antibodies.” <b>ADE-35, P. 55, ll. 17-20.</b></p> <p>“Bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens. In the present case, one of the binding specificities is for the Apo-2, the other one is for any other antigen, and preferably for a cell-surface protein or receptor or receptor subunit.</p> <p>Methods for making bispecific antibodies are known in the art.” <b>ADE-35, P. 55, ll. 23-30.</b></p> <p>“Other modifications of the Apo-2 antibodies are contemplated. For example, it may be desirable to modify the antibodies of the invention with respect to effector function, so as to enhance the therapeutic effectiveness of the antibodies.” <b>ADE-35, P. 57, ll. 16-19.</b></p> <p>“The agonist is preferably administered to the mammal in a carrier. Suitable carriers and their formulations are described in <u>Remington's Pharmaceutical Sciences</u>, 16th ed., 1980, Mack Publishing Co., edited by Oslo et al. Typically, an appropriate amount of a pharmaceutically-acceptable salt is used in the formulation to render the formulation isotonic. Examples of a pharmaceutically-acceptable carrier include saline, Ringer's solution and dextrose solution. The pH of the solution is preferably from about 5 to about 8, and more preferably from about 7 to about 7.5. Further carriers include sustained release</p>

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	<p>preparations such as semipermeable matrices of solid hydrophobic polymers containing the agonist, which matrices are in the form of shaped articles, e.g., films, liposomes or microparticles. It will be apparent to those persons skilled in the art that certain carriers may be more preferable depending upon, for instance, the route of administration and concentration of agonist being administered." <b>ADE-35, P. 59, ll. 9-25.</b></p> <p>"The agonist antibody can be administered to the mammal by injection (e.g., intravenous, intraperitoneal, subcutaneous, intramuscular), or by other methods such as infusion that ensure its delivery to the bloodstream in an effective form. The agonist may also be administered by intratumoral, peritumoral, intralesional, or perilesional routes, to exert local as well as systemic therapeutic effects. Local or intravenous injection is preferred." <b>ADE-35, P. 59, ll. 26-33.</b></p> <p>"Effective dosages and schedules for administering the agonist antibody may be determined empirically, and making such determinations is within the skill in the art. Those skilled in the art will understand that the dosage of agonist that must be administered will vary depending on, for example, the mammal which will receive the agonist, the route of administration, the particular type of agonist used and other drugs being administered to the mammal. Guidance in selecting appropriate doses for antibody agonists is found in the literature on therapeutic uses of antibodies, e.g., <u>Handbook of Monoclonal Antibodies</u>, Ferrone et al., eds., Noyes Publications, Park Ridge, N.J., (1985) ch. 22 and pp. 303-357; Smith et al., <u>Antibodies in Human Diagnosis and Therapy</u>, Haber et al., eds., Raven Press, New York (1977) pp. 365-389. A typical daily dosage of the agonist used alone might range from about 1 µg/kg to up to 100 mg/kg of body weight or more per day, depending on the factors mentioned above." <b>ADE-35, P. 59, l. 34 - P. 60, l. 10.</b></p> <p>"The agonist antibody may also be administered to the mammal in combination with effective amounts of one or more other therapeutic agents or in conjunction with radiation treatment. Therapeutic agents contemplated include chemotherapeutics as well as immunoadjuvants and</p>

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	<p>cytokines. Chemotherapies contemplated by the invention include chemical substances or drugs which are known in the art and are commercially available, such as Doxorubicin, 5-Fluorouracil, Cytosine arabinoside ("Ara-C"), Cyclophosphamide, Thiotepa, Busulfan, Cytosin, Taxol, Methotrexate, Cisplatin, Melphalan, Vinblastine and Carboplatin. The agonist may be administered sequentially or concurrently with the one or more other therapeutic agents. The amounts of agonist and therapeutic agent depend, for example, on what type of drugs are used, the cancer being treated, and the scheduling and routes of administration but would generally be less than if each were used individually." <b>ADE-35, P. 60, ll. 11-26.</b></p> <p>"Following administration of agonist to the mammal, the mammal's cancer and physiological condition can be monitored in various ways well known to the skilled practitioner. For instance, tumor mass may be observed physically or by standard x-ray imaging techniques." <b>ADE-35, P. 60, ll. 27-31.</b></p> <p>"The Apo-2 antibodies of the invention may also be useful in enhancing immune-mediated cell death in cells expressing Apo-2, for instance, through complement fixation or ADCC." <b>ADE-35, P. 60, ll. 32-34.</b></p> <p>"In a further embodiment of the invention, there are provided articles of manufacture and kits containing Apo-2 or Apo-2 antibodies which can be used, for instance, for the therapeutic or non-therapeutic applications described above." <b>ADE-35, P. 61, l. 37 - P. 62, l. 1.</b></p> <p>"Balb/c mice (obtained from Charles River Laboratories) were immunized by injecting 0.5µg/50µl of an Apo-2 ECD immunoadhesin protein (diluted in MPL-TDM adjuvant purchased from Ribi Immunochemical Research Inc., Hamilton, MT) 11 times into each hind foot pad at 3-4 day intervals. The Apo-2 ECD immunoadhesin protein was generated by fusing an extracellular domain sequence of Apo-2 (amino acids 1-184 shown in Fig. 1) to the hinge and Fc region of human immunoglobulin G<sub>1</sub> heavy chain in pRK5 as described previously [Ashkenazi et al., <u>Proc. Natl. Acad. Sci.</u>, <u>88</u>:10535-10539 (1991)]." <b>ADE-35, P. 74, ll. 4-12.</b></p>



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	<p data-bbox="657 279 1437 533">“Of the hybridoma supernatants screened in the ELISA, 22 supernatants tested positive (calculated as approximately 4 times above background). The supernatants testing positive in the ELISA were further analyzed by FACS analysis using 9D cells (a human B lymphoid cell line expressing Apo-2; Genentech, Inc.) and FITC-conjugated goat anti-mouse IgG.” ADE-35, P. 75, ll. 22-27.</p> <p data-bbox="657 575 1437 644">“FACS analysis showed 8/22 supernatants were positive for anti-Apo-2 antibodies.” ADE-35, P. 75, ll. 36-37.</p> <p data-bbox="657 686 1437 863">“Figure 7 shows the FACS staining of 9D cells incubated with one of the Apo-2 antibodies, referred to as 3F11.39.7. As shown in Figure 7, the 3F11.39.7 antibody recognizes the Apo-2 receptor expressed in 9D cells.” ADE-35, P. 76, ll. 1-4.</p> <p data-bbox="657 905 1437 1045">“Hybridoma supernatants and purified antibodies (as described in Example 9 above) were tested for activity to induce Apo-2 mediated 9D cell apoptosis.” ADE-35, P. 76, ll. 11-13.</p> <p data-bbox="657 1087 1437 1415">“As shown in Figure 8, the 3F11.39.7 antibody (in the absence of the goat anti-mouse IgG Fc) induced apoptosis in the 9D cells as compared to the control antibodies. Agonistic activity, however, was enhanced by Apo-2 receptor cross-linking in the presence of the goat anti-mouse IgG Fc (see Figure 9). This enhanced apoptosis (Figure 9) by the combination of antibodies is comparable to the apoptotic activity of Apo-2L in 9D cells (data not shown).” ADE-35, P. 76, ll. 28-35.</p> <p data-bbox="657 1457 1437 1598">“The results are shown in FIG. 11. The Apo-2 antibody 3F11.39.7 bound to Apo-2. The 3F11.39.7 antibody also showed some cross-reactivity to DR4, but not to DcR1 or DcR2.” ADE-35, P. 78, ll. 12-14.</p> <p data-bbox="657 1640 1437 1745">“Purified scFv antibodies (as described in Example 14 above) were tested for ability to induce Apo-2 mediated apoptosis.” ADE-35, P. 85, ll. 13-14.</p> <p data-bbox="657 1787 1437 1852">“As shown in Figures 13C and 14B, the 16E2 and 20E6 antibodies agonistically induced apoptosis in SK-MES-1</p>

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(a) binds to a soluble extracellular domain sequence of an Apo-2 polypeptide consisting of amino acids 54 to 182 of SEQ ID NO:1 and	cells." ADE-35, P. 86, ll. 13-14.
	<p>"In addition to the annexin V-biotin/streptavidin-[S-35] assay described above, scFv antibodies (as described in Example 14 above) were tested for activity to induce Apo-2 mediated apoptosis via assays utilizing crystal violet." ADE-35, P. 86, ll. 17-20.</p>
	<p>"As shown in Figures 13A, 13B, 14A and 14B, the 16E2 and 20E6 antibodies agonistically induced apoptosis in SK-MES-1 cells." ADE-35, P. 87, ll. 15-16.</p>
	<p>"A purified gD-tagged form of 16E2 scFv was tested for ability to induce Apo-2 mediated apoptosis in a crystal violet assay as described in Example 15 above." ADE-35, P. 87, ll. 22-24.</p>
	<p>"The apoptosis assay was performed essentially as described in Example 15(B) above except that samples were serially diluted 1:3 in the plates and the 16E2-gD tagged antibody was tested in addition to two other preparations of 16E2 scFv (referred to as Prep. A and Prep. B in Figure 14C). The results of the assay showing apoptosis induction in SK-MES-1 cells by 16E2-gD antibody are illustrated in Figure 14C." ADE-35, P. 88, ll. 30-36.</p>
	<p>"3F11.39.7 HB-12456 January 13, 1998" ADE-35, P. 89, ll. 8-9.</p>
	<p>"In another embodiment, the invention provides an isolated extracellular domain (ECD) sequence of Apo-2. Optionally, the isolated extracellular domain sequence comprises amino acid residues 54 to 182 of Fig. 1 (SEQ ID NO:1)." ADE-35, P. 8, ll. 25-28.</p>
	<p>"FIG. 2A shows the derived amino acid sequence of a native sequence human Apo-2--the putative signal sequence is underlined, the putative transmembrane domain is boxed, and the putative death domain sequence is dash underlined. The cysteines of the two cysteine-rich domains are individually underlined." ADE-35, P. 10, ll. 10-14.</p>
	<p>"The 'Apo-2 extracellular domain' or 'Apo-2 ECD' refers</p>

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	<p>to a form of Apo-2 which is essentially free of the transmembrane and cytoplasmic domains of Apo-2. Ordinarily, Apo-2 ECD will have less than 1% of such transmembrane and/or cytoplasmic domains and preferably, will have less than 0.5% of such domains. Optionally, Apo-2 ECD will comprise amino acid residues 54 to 182 of Fig. 1 (SEQ ID NO:1) or amino acid residues 1 to 182 of Fig. 1 (SEQ ID NO:1)." <b>ADE-35, P. 13, ll. 30-37.</b></p> <p>"In general, the signal sequence may be a component of the vector, or it may be a part of the Apo-2 DNA that is inserted into the vector. The heterologous signal sequence selected preferably is one that is recognized and processed (i.e., cleaved by a signal peptidase) by the host cell...In mammalian cell expression the native Apo-2 presequence that normally directs insertion of Apo-2 in the cell membrane of human cells in vivo is satisfactory, although other mammalian signal sequences may be used to direct secretion of the protein, such as signal sequences from secreted polypeptides of the same or related species, as well as viral secretory leaders, for example, the herpes simplex glycoprotein D signal." <b>ADE-35, P. 22, l. 29 - P. 23, l. 10.</b></p> <p>"The entire nucleotide sequence of Apo-2 is shown in Figure 1 (SEQ ID NO:2). Clone 27868 (also referred to as pRK5-Apo-2 deposited as ATCC 209021, as indicated below) contains a single open reading frame with an apparent translational initiation site at nucleotide positions 140-142 [Kozak et al., <i>supra</i>] and ending at the stop codon found at nucleotide positions 1373-1375 (Fig. 1; SEQ ID NO:2). The predicted polypeptide precursor is 411 amino acids long, a type I transmembrane protein, and has a calculated molecular weight of approximately 45 kDa. Hydropathy analysis (not shown) suggested the presence of a signal sequence (residues 1-53), followed by an extracellular domain (residues 54-182), a transmembrane domain (residues 183-208), and an intracellular domain (residues 209-411) (Fig. 2A; SEQ ID NO:1). N-terminal amino acid sequence analysis of Apo-2-IgG expressed in 293 cells showed that the mature polypeptide starts at amino acid residue 54, indicating that the actual signal sequence comprises residues 1-53." <b>ADE-35, P. 65, ll. 9-24.</b></p> <p>"(The Apo-2 ECD construct included residues 183 and 184</p>

Proposed Count	Support in Applicants' 10/052,798 Specification
	<p>shown in Figure. 1 to provide flexibility at the junction, even though residues 183 and 184 are predicted to be in the transmembrane region)." ADE-35, P. 66, ll. 20-23.</p> <p>"A soluble Apo-2 ECD immunoadhesin construct was prepared. The Apo-2 ECD (amino acids 1-184 shown in Fig. 1) was fused to the hinge and Fc region of human immunoglobulin G<sub>1</sub> heavy chain in pRK5 as described previously [Ashkenazi et al., <u>Proc. Natl. Acad. Sci.</u>, 88:10535-10539 (1991)]. The immunoadhesin was expressed by transient transfection into human 293 cells and purified from cell supernatants by protein A affinity chromatography, as described by Ashkenazi et al., <u>supra</u>." ADE-35, P. 66, l. 33 - P. 67, l. 2.</p> <p>"Balb/c mice (obtained from Charles River Laboratories) were immunized by injecting 0.5 .mu.g/50 .mu.l of an Apo-2 ECD immunoadhesin protein (diluted in MPL-TDM adjuvant purchased from Ribi Immunochemical Research Inc., Hamilton, MT) 11 times into each hind foot pad at 3-4 day intervals. The Apo-2 ECD immunoadhesin protein was generated by fusing an extracellular domain sequence of Apo-2 (amino acids 1-184 shown in Fig. 1) to the hinge and Fc region of human immunoglobulin G<sub>1</sub> heavy chain in pRK5 as described previously [Ashkenazi et al., <u>Proc. Natl. Acad. Sci.</u>, 88:10535-10539 (1991)]. The immunoadhesin protein was expressed by transient transfection into human 293 cells and purified from cell supernatants by protein A affinity chromatography, as described by Ashkenazi et al., <u>supra</u> (See also Example 2B above)." ADE-35, P. 74, ll. 4-16.</p> <p>"Figure 7 shows the FACS staining of 9D cells incubated with one of the Apo-2 antibodies, referred to as 3F11.39.7. As shown in Figure 7, the 3F11.39.7 antibody recognizes the Apo-2 receptor expressed in 9D cells." ADE-35, P. 76, ll. 1-4.</p> <p>"The results are shown in FIG. 11. The Apo-2 antibody 3F11.39.7 bound to Apo-2. The 3F11.39.7 antibody also showed some cross-reactivity to DR4, but not to DcR1 or DcR2." ADE-35, P. 78, ll. 12-14.</p> <p>"A phage library was selected using soluble biotinylated</p>

Proposed Count	Support in Applicants' 10/052,798 Specification
(b) induces apoptosis in at least one type of mammalian cancer cell <i>in vivo</i> or <i>ex vivo</i> .	antigen and streptavidin-coated paramagnetic beads. The antigen, an Apo-2 ECD immunoadhesin prepared as described in Example 2B above, was biotinylated using IMMUNOPURE NHS-biotin (biotiny-N-hydroxy-succinimide, Pierce) according to manufacturer's instructions." ADE-35, P. 79, ll. 5-10.
	"To evaluate the specificity of each of the scFv clones, ELISA assays were performed to evaluate binding of 16E2, 20E6 and 24C4 to Apo-2 ECD-Ig, DR4-Ig, DcR1-Ig, DcR2-Ig and CD4-Ig (described above and in Example 12)." ADE-35, P. 83, l. 35 - P. 84, l. 2.
	"As illustrated in Figures 12A, 12B and 12C, the ELISA assays showed that each of these antibodies exhibited a relatively high degree of specificity for Apo-2." ADE-35, P. 84, ll. 23-25.
	"The Apo-2 antibodies of the invention have therapeutic utility. Agonistic Apo-2 antibodies, for instance, may be employed to activate or stimulate apoptosis in cancer cells. Accordingly, the invention provides methods for treating cancer using such Apo-2 antibodies." ADE-35, P. 59, ll. 2-6.
	"As shown in Figure 8, the 3F11.39.7 antibody (in the absence of the goat anti-mouse IgG Fc) induced apoptosis in the 9D cells as compared to the control antibodies. Agonistic activity, however, was enhanced by Apo-2 receptor cross-linking in the presence of the goat anti-mouse IgG Fc (see Figure 9). This enhanced apoptosis (Figure 9) by the combination of antibodies is comparable to the apoptotic activity of Apo-2L in 9D cells (data not shown)." ADE-35, P. 76, ll. 28-35.
	"Purified scFv antibodies (as described in Example 14 above) were tested for ability to induce Apo-2 mediated apoptosis." ADE-35, P. 85, ll. 13-14.  "As shown in Figures 13C and 14B, the 16E2 and 20E6 antibodies agonistically induced apoptosis in SK-MES-1 cells." ADE-35, P. 86, ll. 13-14.

Proposed Count	Support in Applicants' 10/052,798 Specification
	<p data-bbox="659 243 1435 422">“In addition to the annexin V-biotin/streptavidin-[S-35] assay described above, scFv antibodies (as described in Example 14 above) were tested for activity to induce Apo-2 mediated apoptosis via assays utilizing crystal violet.” <b>ADE-35, P. 86, ll. 17-20.</b></p> <p data-bbox="659 464 1435 569">“As shown in Figures 13A, 13B, 14A and 14B, the 16E2 and 20E6 antibodies agonistically induced apoptosis in SK-MES-1 cells.” <b>ADE-35, P. 87, ll. 15-16.</b></p> <p data-bbox="659 611 1435 758">“A purified gD-tagged form of 16E2 scFv was tested for ability to induce Apo-2 mediated apoptosis in a crystal violet assay as described in Example 15 above.” <b>ADE-35, P. 87, ll. 22-24.</b></p> <p data-bbox="659 800 1435 1083">“The apoptosis assay was performed essentially as described in Example 15(B) above except that samples were serially diluted 1:3 in the plates and the 16E2-gD tagged antibody was tested in addition to two other preparations of 16E2 scFv (referred to as Prep. A and Prep. B in Figure 14C). The results of the assay showing apoptosis induction in SK-MES-1 cells by 16E2-gD antibody are illustrated in Figure 14C.” <b>ADE-35, P. 88, ll. 30-36.</b></p>

## **APPENDIX J**

### **List of Documentary Exhibits Supporting the Request for Interference**

<b>Designated Exhibit</b>	<b>Description of the Document</b>
<b>ADE-1</b>	Declaration of William Wood, Ph.D.
<b>ADE-2</b>	Declaration of Diane Marschang
<b>ADE-3</b>	Declaration of Avi Ashkenazi, Ph.D.
<b>ADE-4</b>	Declaration of Robert Pitti
<b>ADE-5</b>	Declaration of James Sheridan, Ph.D.
<b>ADE-6</b>	Declaration of Scot Marsters
<b>ADE-7</b>	Email received by William Wood from Dr. Ashkenazi dated prior to March 17, 1997, relating to Incyte sequences.
<b>ADE-8</b>	Email received by William Wood from Dr. Ashkenazi dated prior to March 17, 1997, regarding clone numbers.
<b>ADE-9</b>	Printout of sequences for Incyte clones 2078364 and 1237537.
<b>ADE-10</b>	Draft patent application, draft # 1, dated May 5, 1997.
<b>ADE-11</b>	A Research and Development Information Release Application (referred as a "blue sheet") received on May 5, 1997.
<b>ADE-12</b>	Manuscript titled "A Second Cell Death Receptor For The Cytokine Apo-2 Ligand" authored by Ashkenazi <i>et al.</i> , dated May 5, 1997.
<b>ADE-13</b>	Draft patent application, draft # 2, dated May 13, 1997.

<b>ADE-14</b>	Papers filed with the ATCC depositing a plasmid containing the DNA encoding the Apo-2 receptor dated May 7, 1997.
<b>ADE-15</b>	Facsimile dated May 9, 1997, from Frank P. Simione of the ATTC to Diane Marschang requesting additional information regarding the deposit of pRK5-Apo-2.
<b>ADE-16</b>	Letter dated May 13, 1997, from Diane Marschang to Frank P. Simione indicating the source of the Apo-2 DNA is human.
<b>ADE-17</b>	Letter dated May 15, 1997, from Barbara M. Hailey to Diane Marschang confirming the May 8, 1997, deposit date of the pRK5-Apo-2 construct.
<b>ADE-18</b>	U.S. Patent Application No. 60/046,615 to Ashkenazi <i>et al.</i> , filed on May 15, 1997.
<b>ADE-19</b>	Lab Notebook No. 22265, pages 59-65.
<b>ADE-20</b>	Lab Notebook No. 26610, pages 68-70, 72-78, 80, 83-91, and 96.
<b>ADE-21</b>	Lab Notebook No. 27510, pages 1, 2, 4-5, 8-10, 17, 21-29, 32-34, 36, and 38-42.
<b>ADE-22</b>	Lab Notebook No. 26508, pages 80, 82, 83-85, and 88-93.
<b>ADE-23</b>	Lab Notebook No. 27250, pages 1, 2, 4, 6-8, 12-14, 20-22, 25, 41, 43, 48-49, 51, and 56.
<b>ADE-24</b>	Lab Notebook No. 26865, pages 47, 56, 62, 65-69, 71-73, 76, 77, 87, and 90-96.
<b>ADE-25</b>	Lab Notebook No. 26119, pages 41-54.



<b>ADE-26</b>	Lab Notebook No. 27505, pages 1-6, and 18.
<b>ADE-27</b>	Lab Notebook No. 26577, pages 76-79, 82, 84-86, 88-93, and 95.
<b>ADE-28</b>	Lab Notebook No. 27236, pages 1-9, 11-13, 16-18, 23, 26, 29, 32, 33, and 35.
<b>ADE-29</b>	Lab Notebook No. 26466, pages 60, 71, and 73.
<b>ADE-30</b>	Order form submitted by Scot Marsters to the Genentech Synthetic Oligonucleotide facility describing the PCR primers and DNA probe dated prior to March 17, 1997.
<b>ADE-31</b>	Order form submitted by Scot Marsters to the Genentech Synthetic Oligonucleotide facility describing the PCR primers and DNA probe dated prior to March 17, 1997.
<b>ADE-32</b>	Order form submitted by Scot Marsters to the Genentech Synthetic Oligonucleotide facility describing the PCR primers and DNA probe dated March 19, 1997.
<b>ADE-33</b>	U.S. Patent Application No. 60/074,119, filed on February 9, 1998.
<b>ADE-34</b>	U.S. Patent Application No. 09/079,029, filed on May 14, 1998.
<b>ADE-35</b>	U.S. Patent Application No. 10/052,798, filed on November 2, 2001.
<b>ADE-36</b>	Sequence of entire insert of clone 27868 dated prior to March 17, 1997.
<b>ADE-37</b>	Interference No. 105,380 – Decision-Motions-Bd.R. 125(a) dated March 9, 2007 (Paper No. 95).
<b>ADE-38</b>	Interference No. 105,381 – Decision-Motions-Bd.R. 125(a) dated

	March 26, 2007 (Paper No. 101).
<b>ADE-39</b>	Interference No. 105,361 – Order-Bd.R. 104(a) dated March 28, 2007 (Paper No. 103).

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: )  
CAMELLA W. ADAMS *et al.* ) Docket No.: 22338-00904/P1101R2D1  
Application No. 10/052,798 ) Examiner: Eileen B. O'Hara  
Filed: November 2, 2001 ) Group Art Unit: 1646  
For: Apo-2 RECEPTOR ) Declaration in Support of Request for  
 ) Declaration of Interference  
 ) Under 37 C.F.R. § 41.202  
 ) **Expedited Handling Requested**  
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P.O. Box 1450  
Alexandria, VA 22313-1450

DECLARATION OF WILLIAM WOOD, Ph.D.

**ADE- 1**  
USSN 10/052,798

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## **DECLARATION OF WILLIAM WOOD, Ph.D.**

I, William Wood, declare and state as follows:

### **I. Introduction**

1. I am a citizen of the United States and presently reside in Cupertino, CA.
2. I received my Bachelor of Arts degree in Chemistry from Cornell University in 1970. I received my Ph.D. from the Department of Biochemistry and Molecular Biology at Harvard University in 1977.
3. I have been employed by Genentech Inc., South San Francisco, CA ("Genentech") since 1982.
4. From 1996 to 1998, I was a Staff Scientist in the Molecular Biology Department at Genentech.
5. My current title at Genentech is the Director of Bioinformatics.
6. In this declaration, I provide a description of: 1) my involvement with receiving and managing access to an Incyte sequence database (LifeSeq™); and 2) my interactions with another Genentech scientist, Dr. Avi Ashkenazi, regarding two specific sequences obtained from the Incyte database.
7. In this declaration, I refer to the following documents: 1) two e-mails that I received from Dr. Ashkenazi (**ADE-7** and **ADE-8**) and 2) a printout of sequences for Incyte clones 2078364 and 1237537 (**ADE-9**).
8. I understand that Genentech intends to file this declaration at the United States Patent and Trademark Office to establish activities that occurred prior to March 17, 1997 and up to May 15, 1997. I understand that March 17, 1997, is a date by which another company filed a patent application relating to the Apo-2 receptor (also known as DR5) and that May 15, 1997, is

the date on which Genentech filed a patent application relating to the Apo-2 receptor. Dates preceding March 17, 1997 on documents cited in this declaration have been redacted.

## **II. The Incyte LifeSeq™ Database**

9. Prior to March 17, 1997, I was involved in acquiring Genentech's rights to access Incyte's LifeSeq™ sequence database.

10. Prior to March 17, 1997, I was the person at Genentech primarily responsible for managing access to the Incyte database and maintaining records of sequences identified from the Incyte database (*i.e.*, "banking" the sequences).

## **III. Interactions with Dr. Ashkenazi Regarding Incyte Clones 2078364 and 1237537**

11. I recall informing Dr. Ashkenazi that the Incyte LifeSeq™ database had been successfully incorporated into Genentech's computer network and that it was available for searching.

12. Prior to March 17, 1997, I granted Dr. Ashkenazi access to the Incyte LifeSeq™ database.

13. Prior to March 17, 1997, I understood that Dr. Ashkenazi was searching for novel receptor molecules in the Tumor Necrosis Factor (TNF) family of receptors. I understood that Dr. Ashkenazi was searching for novel receptor sequences based on structural similarities to previously identified receptors in the TNF family (*e.g.*, Apo-3 and Fas), including sequences for 1) cysteine-rich regions in the extracellular domain, 2) a transmembrane domain; and 3) an intracellular region containing a death domain.

14. I recall that prior to March 17, 1997, Dr. Ashkenazi searched the Incyte LifeSeq™ database and identified DNA sequences that he considered to be similar to the sequence of the Apo-3 receptor.

15. Specifically, prior to March 17, 1997, I received an e-mail from Dr. Ashkenazi relating to sequences from the Incyte database. A copy of the e-mail is marked as **ADE-7**. In this e-mail, Dr. Ashkenazi informed me that he planned to clone from “Cluster 75799” two Incyte sequences that were “homologous to the Apo-3 death domain.”

16. Prior to March 17, 1997, I responded to Dr. Ashkenazi’s e-mail (**ADE-7**) and requested Dr. Ashkenazi to provide me the clone numbers corresponding to each of the sequences he intended to clone from Cluster 75799. (**ADE-8**)

17. Prior to March 17, 1997, in response to my request for clone numbers, Dr. Ashkenazi sent me an e-mail identifying clones 2078364 and 1237537 as the sequences he planned to clone from Cluster 75799. A copy of this correspondence is marked as **ADE-8**.

18. For record-keeping, after receiving Dr. Ashkenazi’s e-mails regarding Incyte clones 2078364 and 1237537 and prior to March 17, 1997, I accessed the Incyte LifeSeq™ database and retrieved the sequence data corresponding to Incyte clones 2078364 and 1237537. At that time, I stored a copy of these sequence data in an electronic folder that I created and designated for Incyte sequences identified by Genentech scientists. For the purpose of preparing this declaration, I printed from this electronic folder a copy of the sequence data for Incyte clones 2078364 and 1237537. A copy of this printout is marked as **ADE-9**. At the top of **ADE-9**, the directory information for the files is listed, including the creation dates which are prior to March 17, 1997. The content of the respective sequence files printed below the directory information appears as follows:

```

leu> pwd
/home/ruby/va/Molbio/wiw/incyte/gene6
leu> ls -l
total 24
-rwxr-x--- 1 wiw      Molbio      297  R  ss.INC1237537*
-rwxr-x--- 1 wiw      Molbio      307  ss.INC2078364*
-rwxr-x--- 1 wiw      Molbio      342  ss.gene6.consensus*
leu> more ss.*
::::::::::::
ss.INC1237537
::::::::::::
>1237537      LUNGTUT02      INCYTE
CTTTGACTNCTGGGAGCCGCTCATGAGGAAGTTGGGCCTCATGGACAATGAGATAAAGGT
GGCTAAAGCTGAGGCAGCGGGCCACAGGGACACCTTGTACACGNTGCTGATAAAGTGGGT
CAACAAAACCGGGCGAGATGCCTCTGTCCACACCCTGCTGGATGCCTTGGAGACGCTGGG
AGAGAGACTTGCCAAGCAGANGATTGNGGACCACTTGTTGNGCTCTGGAAAGTTCATGTA
TCTNGAAGGTAATGCAGACT
::::::::::::
ss.INC2078364
::::::::::::
>2078364      ISLTNOT01      INCYTE
TGCTGGTTCCAGCAAATGAAGGTGATCCCACTGAGACTCTGAGACAGTGCTTCGATGACT
TTGCAGACTTGGTGCCCTTTGACTCCTGGGAGCCGCTCATGAGGAAGTTGGGCCTCATGG
ACAATGAGATAAAGGTGGCTAAAGCTGAGGCAGCGGGCCACAGGGACACCTTGTACACGA
TGCTGATAAAGTGGGTCAACAAAACCGGGCGAGATGCCTCTGTCCAAACCCTGCTGGATG
CCTTGGAGACGCTGGGAGAGAGACTTGCCA
::::::::::::
ss.gene6.consensus
::::::::::::
TGCTGGTTCCAGCAAATGAAGGTGATCCCACTGAGACTCTGAGACAGTGCTTCGATGACT
TTGCAGACTTGGTGCCCTTTGACTNCTGGGAGCCGCTCATGAGGAAGTTGGGCCTCATGG
ACAATGAGATAAAGGTGGCTAAAGCTGAGGCAGCGGGCCACAGGGACACCTTGTACACGN
TGCTGATAAAGTGGGTCAACAAAACCGGGCGAGATGCCTCTGTCCACACCCTGCTGGATG
CCTTGGAGACGCTGGGAGAGAGACTTGCCAAGCAGANGATTGNGGACCACTTGTTGNGCT
CTGGAAAGTTCATGTATCTNGAAGGTAATGCAGACT
leu>

```

19. At the time of the above-identified e-mail correspondence between Dr. Ashkenazi and me (**ADE-7** and **ADE-8**), I clearly understood Dr. Ashkenazi's objectives regarding newly identified receptor molecules in the TNF family. My office was in close proximity to Dr. Ashkenazi's office and we often discussed our respective research. Moreover, prior to March 17, 1997, I assisted Dr. Ashkenazi with searches of other, publicly available, genomic databases to further Dr. Ashkenazi's objective of identifying novel receptors in the TNF family. At that time, I understood that upon identifying a novel receptor sequence, Dr. Ashkenazi planned to




conduct cloning and expression experiments as well as to prepare a fusion protein (Apo-2-IgG (immunoglobulin G)) to use in binding studies. Additionally, I understood that Dr. Ashkenazi planned to initiate experiments to prepare antibodies against the novel receptor, including the preparation of agonist antibodies that would specifically bind to the extracellular domain (amino acids 54 to 182) of the receptor, such as the Apo-2 receptor ultimately obtained using clones 2078364 and 1237537, and that would induce apoptosis in a DR5-expressing cell.

20. Specifically, prior to March 17, 1997, I understood that Dr. Ashkenazi had contemplated a method of inducing apoptosis in a DR5-expressing cell which involved exposing the cell to an effective amount of an Apo-2 agonist monoclonal antibody which (a) binds to a soluble extracellular domain sequence (amino acids 54 to 182) of an Apo-2 polypeptide and (b) induces apoptosis in a DR5-expressing cell.

#### **IV. Conclusion**

21. I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of any patents issuing from the above-identified application.

Date: 4/20/07

  
\_\_\_\_\_  
William Wood, Ph.D.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	)
CAMELLA W. ADAMS <i>et al.</i>	) Docket No.: 22338-00904/P1101R2D1
	)
Application No. 10/052,798	) Examiner: Eileen B. O'Hara
	)
Filed: November 2, 2001	) Group Art Unit: 1646
	)
For: Apo-2 RECEPTOR	) <u>Declaration in Support of Request for</u>
	) <u>Declaration of Interference</u>
	) <u>Under 37 C.F.R. § 41.202</u>
	)
	) <b><u>Expedited Handling Requested</u></b>
	)
	)

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COMMISSIONER FOR PATENTS  
P.O. Box 1450  
Alexandria, VA 22313-1450

**DECLARATION OF DIANE MARSCHANG, ESQ.**

**ADE- 2**  
USSN 10/052,798

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## **DECLARATION OF DIANE MARSCHANG, ESQ.**

I, Diane Marschang, declare and state as follows:

### **I. Introduction**

1. In this declaration, I describe my recollections and activities involved with the preparation and filing of provisional application serial number 60/046,615, filed on May 15, 1997 (“the ‘615 application”). My activities included generating drafts of the patent specification, coordinating the deposit of biological material with the ATCC, interacting with Dr. Ashkenazi to discuss the application, reviewing a manuscript provided to me by Dr. Ashkenazi corresponding to his work on the Apo-2 receptor, and filing the ‘615 patent application.

2. In this declaration, I refer to the following documents: a draft patent application dated May 5, 1997 (**ADE-10**); a Research and Development Information Release Application (also referred to as a “blue sheet”) received in the Genentech Legal Department on May 5, 1997 (**ADE-11**); an Apo-2 related manuscript received in the Genentech Legal Department no later than May 5, 1997 (**ADE-12**); a draft patent application dated May 13, 1997 (**ADE-13**); papers filed with the ATCC depositing a plasmid containing the cDNA encoding the Apo-2 receptor dated May 7, 1997 (**ADE-14**); correspondence dated May 9, 1997 from the ATCC requesting additional information regarding the deposit of pRK5-Apo-2 (**ADE-15**); correspondence dated May 13, 1997 to the ATCC regarding the Apo-2 deposit (**ADE-16**); correspondence dated May 15, 1997 from the ATCC confirming the Apo-2 deposit (**ADE-17**); and the specification of the ‘615 patent application, as filed on May 15, 1997 (**ADE-18**).

### **II. Relevant Professional Background**

3. I am a citizen of the United States and presently reside in Edina, Minnesota.

4. I am a registered Patent Attorney before the United States Patent and Trademark Office.

5. I am presently employed by Genentech Inc., South San Francisco, CA ("Genentech") as Senior Patent Counsel.

6. I began my employment with Genentech in 1994 as a patent attorney. My responsibilities included the preparation and prosecution of patent applications, as well as general client counseling activities. One technical area in which I oversaw intellectual property matters was the Tumor Necrosis Factor (TNF) family of ligands and receptors.

### **III. Activities Relating to Apo-2 Receptor**

7. In the course of carrying out my responsibilities relating to the TNF family of ligands and receptors, I became familiar with work conducted by a Genentech scientist named Dr. Avi Ashkenazi.

8. In 1997, I became aware of Dr. Ashkenazi's work relating to a TNF receptor designated as "the Apo-2 receptor." I do not presently recall the specific date on which I first became aware of Dr. Ashkenazi's Apo-2 work, but I believe it could have been as early as March or April of 1997.

9. The earliest documentary evidence that I have located reflecting my interactions with Dr. Ashkenazi regarding the Apo-2 receptor work is dated May 5, 1997.

10. On or about May 5, 1997, the Genentech Legal Department received a Research and Development Information Release Application (RDIRA) submitted by Dr. Ashkenazi.

(ADE-11). The Genentech Legal Department date stamp of May 5, 1997 is located on the upper right corner of the front page of the RDIRA submitted by Dr. Ashkenazi.

11. The RDIRA was an internal approval form used by Genentech scientists prior to submitting a manuscript to a journal or other outside entity for publication.

12. The “Title of Report” line in section 1 of the RDIRA submitted by Dr. Ashkenazi reads “A Second Cell Death Receptor for the Cytokine Apo-2 Ligand.”

13. The “Proposed date of submission” line in section 1 of the RDIRA submitted by Dr. Ashkenazi reads “May 5, 1997.”

14. The “Author submitting for release” line in section 1 of the RDIRA submitted by Dr. Ashkenazi reads “Avi Ashkenazi.”

15. The “name of patent attorney/agent who should review this material” line in section 1 of the RDIRA submitted by Dr. Ashkenazi reads “Diane Marschang” and lists my telephone extension “x5416.”

16. In section 6 of the RDIRA submitted by Dr. Ashkenazi, I recommended that the manuscript be released, noting that a correction to page 6 of the manuscript had been communicated to Dr. Ashkenazi on May 6, 1997. On May 6, 1997, I signed and dated the RDIRA submitted by Dr. Ashkenazi.

17. A copy of Dr. Ashkenazi’s manuscript titled “A Second Cell Death Receptor for the Cytokine Apo-2 Ligand” was attached to the RDIRA submitted by Dr. Ashkenazi. The manuscript has been marked as **ADE-12**.

18. A draft of the ‘615 application (**ADE-10**) contains the following entry at the top of page 1: “Draft # 1- May 5, 1997.” This is a notation that I entered in the draft on May 5, 1997, to reflect the date upon which I completed the first draft of the ‘615 application. The May 5, 1997, draft is 68 pages in length, not including the six figures referenced therein. The first line in the top right corner of the first page of the May 5, 1997, ‘615 application draft reads

“PATENT DOCKET NO. P1101.” Based on the length and content of the May 5, 1997, draft application, I believe that I began work on the draft application prior to May 5, 1997.

19. In the course of preparing the ‘615 application, I coordinated the deposit of a construct containing the cDNA encoding the Apo-2 receptor with the American Type Culture Collection (“ATCC”) in Rockville MD.

20. **ADE-14** contains a cover sheet titled “Shipping Form” and an attached form titled “American Type Culture Collection” and designated in the lower right corner “Form BP/1.”

21. Item number 2 of the ATCC Form BP/1 identifies the deposited strain designation as “pRK5-Apo-2” reflecting my understanding that the material deposited with the ATCC was the Apo-2 construct.

22. Item 19 of the ATCC Form BP/1 provides the additional comment to “Please refer to case no.: P1101” which is the same as the PATENT DOCKET NO. listed at the top of the first page of the May 5, 1997, draft of the ‘615 application.

23. The submission form was signed at the bottom by Janet Hasak on May 7, 1997. Janet Hasak was a patent attorney in the Genentech patent department. I also entered my handwritten initials next to the “Typed Name” entry at item 19.

24. On May 9, 1997, I received a facsimile from Frank Simione of the ATCC, requesting that I provide additional information regarding the deposit of pRK5-Apo2 with reference to case number P1101. The date of May 9, 1997 is reflected at the bottom of the letter by the facsimile date stamp. (**ADE-15**).

25. On May 13, 1997, I responded to the May 9, 1997, ATCC letter and advised the ATCC that the source of the Apo-2 DNA is human and that the pRK5 plasmid was derived from *E. coli*. (**ADE-16**).

26. **ADE-13** is a subsequent draft of the '615 application that I prepared.

27. The following entry is at the top of page 1 of **ADE-13** "Draft #2 – May 13, 1997." This is a notation that I entered in the draft on May 13, 1997, to reflect the date upon which I completed the second draft of the '615 application. The May 13, 1997, draft is 74 pages in length, not including the six figures referenced therein. The first line in the top right corner of the first page of the May 13, 1997, '615 application draft reads "PATENT DOCKET NO. P1101."

28. I received a letter dated May 15, 1997, by facsimile on May 16, 1997, from Barbara Hailey, the ATCC administrator of the ATCC Patent Depository, which confirmed the May 8, 1997, deposit date of the pRK5-Apo-2 construct. (**ADE-17**).

**IV. Filing of the '615 Application**

29. On May 15, 1997, I filed the '615 application with the United States Patent and Trademark Office. (**ADE-18**).

**V. Conclusion**

30. I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of any patents issuing from the above-identified application.

Date: April 19, 2007

Diane Marschang  
Diane Marschang, Esq.



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: )  
CAMELLA W. ADAMS *et al.* ) Docket No.: 22338-00904/P1101R2D1  
Application No. 10/052,798 )  
Filed: November 2, 2001 ) Examiner: Eileen B. O'Hara  
For: Apo-2 RECEPTOR ) Group Art Unit: 1646  
 ) Declaration in Support of Request for  
 ) Declaration of Interference  
 ) Under 37 C.F.R. § 41.202  
 ) Expedited Handling Requested  
 )  
 )

---

COMMISSIONER FOR PATENTS  
P.O. Box 1450  
Alexandria, VA 22313-1450

DECLARATION OF DR. AVI ASHKENAZI, Ph.D.

**ADE- 3**  
USSN 10/052,798

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## **DECLARATION OF DR. AVI ASHKENAZI, Ph.D.**

I, Avi Ashkenazi, declare and state as follows:

### **I. Introduction and Background**

1. I am a citizen of the United States and presently reside in San Mateo, CA.
2. I received both my Bachelor's degree in Biochemistry, with honors, in 1983, and my Ph.D in Biochemistry in 1986, from Hebrew University in Israel.
3. I have been employed by Genentech Inc., South San Francisco, CA ("Genentech") since 1988, initially as a post-doctoral fellow in the Molecular Biology Group, and later during 1996-1998 as a Senior Scientist, and the Interim Director of the Molecular Oncology Group at Genentech.
4. I am presently employed by Genentech as a Staff Scientist and as a Director of Research in the Molecular Oncology Group.
5. In this declaration, I provide a description of my activities and the activities of those acting on my behalf with regard to the development of monoclonal agonist and antagonist antibodies that specifically bind to the extracellular domain of the Apo-2 receptor, as reflected in a patent application having serial number 60/046,615 ("the '615 application"), filed on May 15, 1997, and naming me as an inventor. Specifically, in this declaration, I provide the following:
  - a) A brief overview of the technology relating to the Apo-2 receptor;
  - b) A brief overview of the individuals with whom I interacted at Genentech regarding Apo-2 prior to May 15, 1997, the filing date of the '615 application; and
  - c) A description of my activities, and activities conducted on my behalf, relating to

the work described in the '615 patent application.

6. In this Declaration I refer to Ashkenazi Documentary Exhibits ("ADE") 8, 10-13, 18, and 19.

7. I understand that Genentech intends to submit this declaration to the United States Patent and Trademark Office to establish activities that occurred prior to March 17, 1997 and up to May 15, 1997. I understand that March 17, 1997, is a date by which another company filed a patent application relating to the Apo-2 receptor (also known as DR5) and that May 15, 1997, is the date on which Genentech filed the '615 patent application relating to the Apo-2 receptor.

8. Certain work conducted by me, or on my behalf, directed to identifying and characterizing the Apo-2 receptor occurred prior to March 17, 1997, and is described in such terms, while other work occurred after March 17, 1997, and is described with reference to the specific dates. Dates prior to March 17, 1997, in documents cited herein have been redacted.

## **II. Overview of Apo-2 Work**

9. In the mid-1990's, I had a research goal to identify new receptors and ligands in the Tumor Necrosis Factor ("TNF") family. My research resulted in the identification and characterization of a novel TNF receptor, which I ultimately designated "Apo-2." My research included the preparation of antibodies against Apo-2, including agonist antibodies that specifically bind to the extracellular domain (amino acids 54 to 182) of the Apo-2 receptor and induce apoptosis in a DR5-expressing cell.

10. The Apo-2 receptor is a transmembrane receptor having 1) cysteine-rich regions in the extracellular domain, 2) a transmembrane domain; and 3) an intracellular region containing a death domain.

11. I first identified a portion of the Apo-2 receptor molecule prior to March 17, 1997, using the LifeSeq™ sequence database, which Genentech had licensed from Incyte Pharmaceuticals, Inc. (“Incyte”).

12. Prior to March 17, 1997, I identified the complete cDNA sequence of the Apo-2 receptor. Based on the cDNA sequence, I was also able to identify the amino acid sequence of the Apo-2 receptor prior to March 17, 1997.

13. Prior to March 17, 1997, I was aware of the sequence of the putative extracellular, transmembrane and intracellular domains of the Apo-2 receptor. I further understood that the intracellular domain of the Apo-2 receptor contained a “death domain” sequence that was similar to a structural feature found in the Apo-3 receptor.

14. Prior to March 17, 1997, based on the available information relating to the Apo-2 receptor, I fully contemplated the idea that monoclonal antibodies could be prepared that would specifically bind to the extracellular domain of the Apo-2 receptor and that such antibodies could be agonists of the Apo-2 receptor. Prior to March 17, 1997, I understood that a possible agonistic effect of such an antibody would be the induction of apoptosis in the cell, but that other agonistic functions were also possible.

15. Specifically, prior to March 17, 1997, I fully contemplated a method of inducing apoptosis in a DR5-expressing cell which involved exposing the cell to an effective amount of an Apo-2 agonist monoclonal antibody which (a) binds to a soluble extracellular domain sequence (amino acids 54 to 182) of an Apo-2 polypeptide and (b) induces apoptosis in a DR5-expressing cell.

### **III. Overview of Individuals Working on My Behalf**

16. There are several individuals who worked on my behalf to characterize the new

receptor I identified and toward the filing of the '615 patent application.

17. A Genentech scientist, Dr. William Wood, assisted me with receiving training and obtaining access to the Incyte LifeSeq™ sequence database. After searching the Incyte sequence database and identifying expressed sequence tags (ESTs) of interest, I conveyed the relevant information to Dr. Wood.

18. Several Genentech scientists in my laboratory assisted me with the cloning and initial characterization of the full length Apo-2 receptor molecule, including Scot Marsters, a Senior Research Associate; Dr. James Sheridan, a post-doctoral fellow; Robert Pitti, a Senior Research Associate; and Maya Skubatch, a Temporary Research Associate. Lakshmi Ramakrishnan, a Research Associate who worked in a different Genentech laboratory, assisted with the isolation of clones containing the full-length cDNA encoding the Apo-2 receptor.

19. The initial activities directed to identifying and characterizing the Apo-2 receptor are described in Examples 1 through 8 of the '615 application. Generally, the work done by me or on my behalf relating to the Apo-2 receptor, as reflected in the specification of the '615 application (**ADE-18**), involved the following:

- a. Identifying, isolating, cloning, and sequencing the DNA sequence encoding the Apo-2 receptor (Example 1);
- b. Constructing and expressing Apo-2-Flag, Apo-2 IgG, and Apo-2-GST fusion proteins for use in binding studies (Examples 2, 3, and 4);
- c. Overexpressing the DNA encoding the Apo-2 receptor in a cell-based system to determine whether the receptor induces apoptosis (Example 4);
- d. Binding studies using the Apo-2 fusion proteins and the Apo-2 ligand (Example 5);

- e. Evaluating whether the newly identified Apo-2 receptor activates the NFκB pathway (Example 6);
- f. Evaluating tissue localization of Apo-2 mRNA via Northern blot analyses (Example 7); and
- g. Conducting chromosomal mapping experiments to map the location of the gene encoding the Apo-2 receptor and the gene encoding DR4 (Example 8).

20. Finally, I also worked with patent counsel for Genentech, Diane Marschang, and assisted her in the preparation of the '615 patent application. As reflected, for example, at page 10, lines 3-5, and page 56, lines 22-23, of the '615 application, I contemplated the preparation of antibodies, including agonist antibodies, that bind to the extracellular domain of the Apo-2 receptor.

#### **IV. Description of Activities Preceding the Filing of the '615 Application**

##### **A. Identifying Clones from the Incyte LifeSeq™ Sequence Database**

21. The initial work I conducted toward isolating and characterizing the Apo-2 receptor, and developing monoclonal agonist antibodies that specifically bind the extracellular domain (amino acids 54 to 182) of the Apo-2 receptor and induce apoptosis in a DR5-expressing cell, involved searching a sequence database that Genentech licensed from Incyte.

22. Incyte was a company in the business of sequencing portions of the human genome. Incyte maintained a proprietary computer database of such sequences and licensed the database to Genentech.

23. The Incyte LifeSeq™ sequence database is made up of DNA sequences referred to as “expressed sequence tags” (“ESTs”) that were generated primarily from the five prime ends

of a collection of cDNA clones.

24. Dr. William Wood worked in the Molecular Biology Group at Genentech and was involved with providing Genentech scientists with access to the Incyte LifeSeq™ database. Dr. Wood also maintained records of the sequences identified from the Incyte database by Genentech scientists.

25. Prior to March 17, 1997, Dr. Wood provided me with access to the Incyte database.

26. Prior to March 17, 1997, I conducted searches of the Incyte database for sequences having structural similarities to the Apo-3 receptor.

27. I identified two EST sequences from the Incyte database, *i.e.*, Incyte clone numbers 2078364 and 1237537, that ultimately led to the isolation and characterization of the Apo-2 receptor, as well as the development of agonist antibodies that specifically bind to the extracellular domain (amino acids 54 to 182) of the Apo-2 receptor and induce apoptosis in a DR5-expressing cell.

28. Prior to March 17, 1997, as reflected on page 59 of my notebook 22265 (**ADE-19**, p. 59), “I found a set of 2 Incyte sequences that show homology to the death domains of Apo-3 and of other TNF receptor family members.” In the right margin of page 59 of notebook 2265, I noted that the Incyte sequences were obtained from Cluster 75799.

29. Below my notation, I attached a copy of the printout of the query that I used to search the Incyte database and which resulted in the identification of Incyte clones 2078364 and 1237537. In the image of the entry below, the “Query” amino acid sequence is from the Apo-3 death domain and the “Sbjct” amino acid sequence is the corresponding sequence of the



respective Incyte clone. The sequence data located between the "Query" and "Subject" data reflects consensus sequence.

```

>2078364 INCTMOT01      INCYTE
      Length = 270
      Plus Strand HSPs:
      score = 85 (19.4 bits), Expect = 0.00067, P = 0.00067
      Identities = 17/43 (39%), Positives = 23/43 (53%), Frame = +3
      Query:      1 DAVPARKNKEFVRIIGLREARIEAVNYEIGRFRDQGYENLKNV 43
                   D YP  N+  +R LGL + EI+  + E   RD Y XL IW
      Subject:    66 DLVPTDSNEPLQKIGLKNREIKVAKAALAGHRTILTYNLKN 194

>1215337 LONGT0102      INCYTE
      Length = 260
      Plus Strand HSPs:
      score = 85 (11.7 bits), Expect = 0.44, P = 0.57
      Identities = 13/36 (36%), Positives = 19/36 (52%), Frame = +2
      Query:      10 WEEFVRIIGLREARIEAVNYEIGRFRDQGYENLKNV 43
                   N+  +R LGL + EI+  + E   RD Y L +W
      Subject:    11 WEPNKKIGLKNREIKVAKAALAGHRTILTYNLKN 118

```

30. Below is a more legible copy of the information contained in my notebook entry from above.

>2078364 ISLTNOT01 INCYTE

Length = 270

Plus Strand HSPs:

Score = 85 (39.6 bits), Expect = 0.00067, P = 0.00067  
Identities = 17/43 (39%), Positives = 23/43 (53%), Frame = +3

Query: 3 DAVPARRWKEFVRTLGLREAEIEAVEVEIGRFRDQQYEMLKRW 45  
D VP W+ +R LGL + EI+ + E RD Y ML +W  
Sbjct: 66 DLVPFDSWEPLMRKLGMDNEIKVAKAEAAGHRDTLYTMLIKW 194

>1237537 LUNGTUT02 INCYTE

Length = 260

Plus Strand HSPs:

Score = 68 (31.7 bits), Expect = 0.46, P = 0.37  
Identities = 13/36 (36%), Positives = 19/36 (52%), Frame = +2

Query: 10 WKEFVRTLGLREAEIEAVEVEIGRFRDQQYEMLKRW 45  
W+ +R LGL + EI+ + E RD Y L +W  
Sbjct: 11 WEPLMRKLGMDNEIKVAKAEAAGHRDTLYTXLIKW 118

31. Next, prior to March 17, 1997, I aligned the sequences of Incyte clones 2078364 and 1237537 to identify conserved regions, which would later be used by Scot Marsters to design PCR primers to isolate and clone the full length sequence from a human pancreas cDNA library. I attached a copy of this sequence alignment in the middle of page 59 of my notebook 22265, an image of which is provided below.

R

/home/ruby/va/Molbio/aa/INER/INERs/incytes/aa.out

2078364 1 T G C T G G T T C C A G C A A A T G A A G G T G A T C C C A C T G A G A C T C T G A G A C A G T G C

2078364 51 T T C G A T G A C T T T G C A G A C T T G G T G C C C T T T G A C T C C T G G G A G C C G C T C A T  
1237537 1 . . . . . C T T T G A C T N C T G G G A G C C G C T C A T

2078364 101 G A G G A A G T T G G G C C T C A T G G A C A A T G A G A T A A A G G T G G C T A A A G C T G A G G  
1237537 25 G A G G A A G T T G G G C C T C A T G G A C A A T G A G A T A A A G G T G G C T A A A G C T G A G G

2078364 151 C A G C G G G C C C A C A G G G A C A C C T T G T A C A C G A T G C T G A T A A A G T G G G T C A A C  
1237537 75 C A G C G G G C C C A C A G G G A C A C C T T G T A C A C G N T G C T G A T A A A G T G G G T C A A C

2078364 201 A A A A C C G G G C G A G A T G C C T C T G T C C A A A C C C T G C T G G A T G C C T T G G A G A C  
1237537 125 A A A A C C G G G C G A G A T G C C T C T G T C C A C A C C C T G C T G G A T G C C T T G G A G A C

2078364 251 G C T G G G A G A G A G A C T T G C C A  
1237537 175 G C T G G G A G A G A G A C T T G C C A A G C A G A N G A T T G H G G A C C A C T T G T T G N G C T

1237537 225 C T G G A A A G T T C A T G T A T C T N G A A G G T A A T G C A G A C T

32. Below is a more legible copy of the information contained in my notebook entry from above.

/home/ruby/va/Molbio/aa/INER/INERs/incytes/aa.out

2078364 1 T G C T G G T T C C A G C A A A T G A A G G T G A T C C C A C T G A G A C T C T G A G A C A G T G C

2078364 51 T T C G A T G A C T T T G C A G A C T T G G T G C C C T T T G A C T C C T G G G A G C C G C T C A T  
1237537 1 . . . . . C T T T G A C T N C T G G G A G C C G C T C A T

2078364 101 G A G G A A G T T G G G C C T C A T G G A C A A T G A G A T A A A G G T G G C T A A A G C T G A G G  
1237537 25 G A G G A A G T T G G G C C T C A T G G A C A A T G A G A T A A A G G T G G C T A A A G C T G A G G

2078364 151 C A G C G G G C C C A C A G G G A C A C C T T G T A C A C G A T G C T G A T A A A G T G G G T C A A C  
1237537 75 C A G C G G G C C C A C A G G G A C A C C T T G T A C A C G N T G C T G A T A A A G T G G G T C A A C

2078364 201 A A A A C C G G G C G A G A T G C C T C T G T C C A A A C C C T G C T G G A T G C C T T G G A G A C  
1237537 125 A A A A C C G G G C G A G A T G C C T C T G T C C A C A C C C T G C T G G A T G C C T T G G A G A C

2078364 251 G C T G G G A G A G A G A C T T G C C A  
1237537 175 G C T G G G A G A G A G A C T T G C C A A G C A G A N G A T T G H G G A C C A C T T G T T G N G C T

1237537 225 C T G G A A A G T T C A T G T A T C T N G A A G G T A A T G C A G A C T

33. Prior to March 17, 1997, I prepared a consensus sequence and attached a copy to the bottom of page 59 of my notebook 22265 (ADE-19, p. 59), an image of which is provided below.

```

R
/tnfr/tnfrs/ncytes/ss.conlDD
length: 336
1 TGCTGGTTCC AGCAAATGAA GGTGATCCCA CTGAGACTCT GAGACAGTGC TTGATGACT TTGCAGACTT GGTGCCCTTT GACTNCTGGG AGCCGCTCAT
ACGACCAAGG TCGTTTACTT CCCTAGGGT GACTCTGAGA CTCTGTCAGG AAGCTACTGA AACGTCTGAA CCACGGGAAA CTGANGACCC TCGGCGAGTA
31 LeuValPr oAlaAsnGlu GlyAspProT hrGluThrLe uArgGlnCys PheAspAspP heAlaAspLe uValProPhe AspUnkTrpC luProLeuMet
101 GAGGAAGTTG GGCCTCATGG ACAATGAGAT AAAGTGGCT AAAGCTGAGG CAGCGGGCCA CAGGGACACC TTGTACACGN TGCTGATAAA GTGGGTCAAC
CTCTTCAAC CCGGAGTACC TGTTACTCTA TTCCACCGA TTTCGACTCC GTCCCGCGGT GTCCCTGTGG AACATGTGCN ACGACTATTT CACCCAGTTG
34 ArgLysLeu GlyLeuMeta sPAsnGluIl eLysValAla LysAlaGluA laAlaGlyHi sArgAspThr LeuTyrThrU nkLeuIleLy sTrpValAsn
201 AAAACCGGGC GAGATGCCCTC TGTCACACCC CTGCTGGATG CTTGGAGAC GCTGGGAGAG AGACTTGCCA AGCAGANGAT TGNGGACCAC TTGTGNGCT
TTTGGCCCC CTCTACGGAG ACAGGTGTGG GACGACCTAC GGAACCTCTG CGACCTCTC TCTGAACGGT TCGTCTNCTA ACNCTGCTG AACAAACNCGA
67 LysThrGlyA rgAspAlaSe rValHisThr LeuLeuAspA laLeuGluTh rLeuGlyGlu ArgLeuAlaL ysGlnUnkIl eUnkAspHis LeuLeuUnkSer
301 CTGAAAGTT CATGTATCTN GAAGGTAATG CAGACT
GACCTTTCAA GTACATAGAN CTTCATTAC GTCTGA
101 GlyLysPh eMetTyrLeu GluGlyAsnA laAsp

```

34. Below is a more legible copy of the information contained in my notebook entry from above.

```

< /home/ruby/va/Molbio/aa/TNFR/TNFRs/ncytes/ss.conlDD
< length: 336

```

```

1 TGCTGGTTCC AGCAAATGAA GGTGATCCCA CTGAGACTCT GAGACAGTGC TTGATGACT TTGCAGACTT GGTGCCCTTT GACTNCTGGG AGCCGCTCAT
ACGACCAAGG TCGTTTACTT CCCTAGGGT GACTCTGAGA CTCTGTCAGG AAGCTACTGA AACGTCTGAA CCACGGGAAA CTGANGACCC TCGGCGAGTA
1 LeuValPr oAlaAsnGlu GlyAspProT hrGluThrLe uArgGlnCys PheAspAspP heAlaAspLe uValProPhe AspUnkTrpC luProLeuMet

101 GAGGAAGTTG GGCCTCATGG ACAATGAGAT AAAGTGGCT AAAGCTGAGG CAGCGGGCCA CAGGGACACC TTGTACACGN TGCTGATAAA GTGGGTCAAC
CTCTTCAAC CCGGAGTACC TGTTACTCTA TTCCACCGA TTTCGACTCC GTCCCGCGGT GTCCCTGTGG AACATGTGCN ACGACTATTT CACCCAGTTG
34 ArgLysLeu GlyLeuMeta sPAsnGluIl eLysValAla LysAlaGluA laAlaGlyHi sArgAspThr LeuTyrThrU nkLeuIleLy sTrpValAsn

201 AAAACCGGGC GAGATGCCCTC TGTCACACCC CTGCTGGATG CTTGGAGAC GCTGGGAGAG AGACTTGCCA AGCAGANGAT TGNGGACCAC TTGTGNGCT
TTTGGCCCC CTCTACGGAG ACAGGTGTGG GACGACCTAC GGAACCTCTG CGACCTCTC TCTGAACGGT TCGTCTNCTA ACNCTGCTG AACAAACNCGA
67 LysThrGlyA rgAspAlaSe rValHisThr LeuLeuAspA laLeuGluTh rLeuGlyGlu ArgLeuAlaL ysGlnUnkIl eUnkAspHis LeuLeuUnkSer

301 CTGAAAGTT CATGTATCTN GAAGGTAATG CAGACT
GACCTTTCAA GTACATAGAN CTTCATTAC GTCTGA
101 GlyLysPh eMetTyrLeu GluGlyAsnA laAsp

```



/home/ruby/va/Molbio/aa/TNFR/TNFRs/incytes/aa.out

```

hApo3      1 MEQRPRGCAAVAAALLLVLLGARAQGGTRSPRCDGDFHKKIGLFCCRG
hApo3      51 GPAGHYLKAPCTEPCGNSTCLVCPQDTFLAWENHHNSECARCQACDEQAS
hApo3     101 QVALENCSAVADTRCGCKPGWFVEGQVSQCVSSSPFYCQPCLDGALHRH
hApo3     151 TRLLCSRRDTCGTCLPGFYEHGDCVSCPTSTLGSCPERCAAVCGWRQM
hApo3     201 FWVQVLLAGLVVPLLLGATLTYYRHCWPHKPLVTADAEAGMEALTPPPAT
hApo3     251 HLSPLDSAHTLLAPPDSSEKICTVQLVGN SWT PGYPETQEALCPQVTWSW
hApo3     301 DQLPSRALGPAAAPT LSPESPAGSPAMMLQPGPQLYDVMDAVPARRWKEF
con1DD      1 . . . . . LVPAN-EGDPTETLRQC--FDDFADLVPFDXWEPL
hApo3     351 VRTLGLREAEIEAVEVEIIGRFRDQQYEMLKRW--RQQQPAGLGAVYAAALE
con1DD     33 MRKLGLMDNEIKVAKAEAAAGHRDITLYTXLIKWVNKTGRDASVHTLLDALE
hApo3     399 RMGLDGCVEDLRSLQRGP
con1DD     83 TLGERLAKQXIXDHLXSGKFMYLEGNA D

```

38. Beneath the sequence comparison between hApo3 and con1DD, I attached a comparison of amino acid sequences of death domains from the TNF receptors Apo-3, TNFR1, and CD95, and the adaptor molecules FADD, TRADD, RIP and Reaper to the amino acid sequence of con1DD1 (handprinted). I attached a printout of this comparison to the middle of page 60 of my notebook 22265. In the printout of the results from this comparison, identical amino acid sequences are marked with boxes. An image of the printout is provided below.

		FAD	V	P	F	D	X	N	E	P	H	A	C	S	L	H	D	N	E	I	K	V	A	K	A	E	A	G	--	H	A	D	T	L	Y	T		
Apo-3	338	V	K	D	A	P	A	R	S	E	T	F	V	T	G	R	E	E	E	A	V	E	V	S	I	R	--	P	R	S	O	Q	Y	E				
TNFR1	333	V	V	E	P	P	L	R	E	E	V	R	E	G	S	S	H	E	O	R	L	E	L	N	G	R	--	C	L	E	Z	A	Q	K	S			
CD95	220	I	A	G	V	H	L	S	O	V	R	O	F	V	E	N	D	V	N	E	X	E	D	E	K	N	D	N	V	O	D	T	A	E	Q	K	V	
FADD	104	I	C	D	N	I	O	--	D	E	R	R	E	A	O	K	V	S	E	T	T	I	O	S	Z	D	R	Y	P	R	N	--	L	T	E	R	V	R
TRADD	211	H	R	P	A	S	L	--	D	O	O	T	E	A	S	V	H	K	K	R	V	O	R	--	S	L	O	R	G	C	R	A	L	S	P	A	L	D
RIP	291	I	R	E	N	L	G	--	H	E	N	C	A	E	S	D	O	T	O	S	A	D	E	T	D	H	D	Y	E	R	D	G	L	K	E	K	V	E
Reaper	21																																					
Apo-3	378	K	E	I	E	R	O	U	O	P	--	K	O	L	G	A	V	A	T	V	R	H	S	D	O	C	V	S	O	L	R	S						
TNFR1	374	E	A	T	R	A	T	T	E	R	E	T	L	E	L	O	R	V	I	R	D	N	D	--	S	O	C	E	D	I	E	S						
CD95	261	L	R	N	H	O	L	H	--	K	K	E	A	Y	D	I	K	D	K	K	A	N	I	C	T	L	A	--	E	X	I	O	T					
FADD	144	S	N	I	K	T	--	K	E	N	T	A	H	V	G	I	R	S	C	--	--	O	H	N	E	V	A	D	L	V								
TRADD	251	S	A	Y	E	Y	E	--	L	Y	E	O	A	F	O	L	E	R	R	F	V	--	Q	A	E	G	R	A	T	E	O	R	L	V	E			
RIP	332	S	O	R	E	V	H	E	--	I	X	G	T	O	R	J	A	O	--	H	Q	C	--	S	R	I	D	L	S	S	L	T						
Reaper	24	F	E	A	T	V	E	L	E	T	L	N	O	T	S	C	H	P	K	T	O	R	K	S	Q	K	Y	R	K	P								

39. Once I identified the partial sequence having structure similarity to the Apo-3 sequence, I contemplated conducting a variety of experiments, including: 1) obtaining the full length sequence of the molecule exhibiting structural similarity to Apo-3, 2) conducting expression and functional assays, 3) identifying ligand(s) that bind(s) to the receptor molecule, and 4) initiating experiments to generate antibodies, including agonist antibodies that specifically bind to the extracellular domain (amino acids 54 to 182) of the newly identified receptor and induce apoptosis in a DR5-expressing cell.

40. After identifying clones 2078364 and 1237537 from the Incyte database, I provided Dr. Wood with the relevant Cluster number and clone numbers. Specifically, prior to March 17, 1997, I sent an e-mail to Dr. Wood relating to sequences from the Incyte database. I attached a copy of my e-mail to the top of page 61 in my notebook 22265 (ADE-19, p. 61). In this e-mail, I informed Dr. Wood that I planned to clone from "Cluster 75799" two Incyte sequences that were "homologous to the Apo-3 death domain."

41. Prior to March 17, 1997, Dr. Wood responded to my e-mail and requested me to provide him with the clone numbers corresponding to each of the sequences I intended to clone from Cluster 75799. (ADE-8).

42. Prior to March 17, 19987, in response to Dr. Wood's request for clone numbers, I sent Dr. Wood an e-mail identifying clones 2078364 and 1237537 as the sequences I planned to clone from Cluster 75799. A copy of this correspondence is marked as **ADE-8**.

43. After identifying clones 2078364 and 1237537 from the Incyte database , I also instructed Mr. Marsters to obtain the full sequence of the molecule having the observed homology with the Apo-3 receptor. On the lower half of page 60 of my notebook 22265 (**ADE-19**, p. 60), beneath the various sequence comparisons, I noted that "I asked Scot Marsters to design probes/primers in order to clone out the full-length cDNA." I further noted on page 60 of my notebook that "This appears clearly to be a novel death domain containing sequence."

44. I also guided Mr. Marsters in experiments designed to evaluate the signaling and binding characteristics of the molecule.

45. Prior to March 17, 1997, I noted on page 62 of my notebook 22265 (**ADE-19**, p. 62) that "Positive phage clones have been identified in two libraries, pancreas and \_\_\_\_\_, using a probe based on the death domain-like Incyte sequence. One clone of each library was sent to sequencing."

46. Prior to March 17, 1997, I noted on page 63 of my notebook 22265 (**ADE-19**, p. 63) that "Today, we obtained evidence for activation of apoptosis by the new death domain protein. Scot transected 293 cells with control pRK5 of phage clone subcloned to pRK5." Beneath my notations, I attached two photographs to page 63 of my notebook. The first is a photograph of cells transfected with pRK5 control DNA. The second is a photograph of cells transfected with the pRK5 vector and the cDNA encoding the death domain containing protein.

47. Prior to March 17, 1997, I noted on page 64 of my notebook 22265 (**ADE-19**, p. 64) that "We obtained full-length sequence of the cDNA." Immediately below my notation on



[illegible]

```

1503 CAGATGACG GATGACGACG AAGATGACG ATGACGACG AGCAGTGGG TGGACGACG GATGACGACG GATGACGACG GATGACGACG GATGACGACG
GATGACGACG GATGACGACG GATGACGACG GATGACGACG GATGACGACG GATGACGACG GATGACGACG GATGACGACG GATGACGACG GATGACGACG
501 NISHLTHR 1YTHRDLAR 2AGHSLRPR 3ICGLNHLN 4AFZOVALL 5POLYTHROR 6YSLHARPHS 7SLKHLKSL 8ALALAPHE 9LEUC*ALAOLN
THRO*PRO VALLEUOLUO 10THLEAPR 11SERASHIL 12THROTRPH 13TOLUKHSL 14VALTRPHE 15SLCYSYTH 16PHIS*YIPH 17TYL*YLEU
NISHLAR 18TYL*YLEU 19LALSLAR 20LALSLAR 21LALSLAR 22LALSLAR 23LALSLAR 24LALSLAR 25LALSLAR 26LALSLAR 27LALSLAR 28LALSLAR
1601 AATGTRTAA TANGACACG ATGAAATGT CTGATCAT 00CTTGCG GTACTTTAG ATT*00TTT 00ATGTCAT 0TTF/CACAG CACTTTTAA
TTACTTATT ATTCCTTGA TACCTTTGA GACCTTTGA GATGACGACG GATGACGACG GATGACGACG GATGACGACG GATGACGACG GATGACGACG
535 CYS*YIPH 1YTHRDLAR 2AGHSLRPR 3ICGLNHLN 4AFZOVALL 5POLYTHROR 6YSLHARPHS 7SLKHLKSL 8ALALAPHE 9LEUC*ALAOLN
AANVALLE 10ALAPHE 11TYL*YLEU 12TYL*YLEU 13TYL*YLEU 14TYL*YLEU 15TYL*YLEU 16TYL*YLEU 17TYL*YLEU 18TYL*YLEU 19TYL*YLEU
METOC*OC 00*0LYNLT 20*0LYNLT 21*0LYNLT 22*0LYNLT 23*0LYNLT 24*0LYNLT 25*0LYNLT 26*0LYNLT 27*0LYNLT 28*0LYNLT
1701 TCGTAAATG AATGCTTTT 2TATTATT 00CTTGAC 00CTTGAC 00CTTGAC 00CTTGAC 00CTTGAC 00CTTGAC 00CTTGAC 00CTTGAC
AGATTTACAT 2TATTATT 00CTTGAC 00CTTGAC 00CTTGAC 00CTTGAC 00CTTGAC 00CTTGAC 00CTTGAC 00CTTGAC
540 PROANVAL AANVALLE 10ALAPHE 11TYL*YLEU 12TYL*YLEU 13TYL*YLEU 14TYL*YLEU 15TYL*YLEU 16TYL*YLEU 17TYL*YLEU 18TYL*YLEU
LEUC*OC 19TYL*YLEU 20TYL*YLEU 21TYL*YLEU 22TYL*YLEU 23TYL*YLEU 24TYL*YLEU 25TYL*YLEU 26TYL*YLEU 27TYL*YLEU
DAROC*YIPH 28TYL*YLEU 29TYL*YLEU 30TYL*YLEU 31TYL*YLEU 32TYL*YLEU 33TYL*YLEU 34TYL*YLEU 35TYL*YLEU
1801 ACCGCGAGG 00CTTGACG 00CTTGACG 00CTTGACG 00CTTGACG 00CTTGACG 00CTTGACG 00CTTGACG 00CTTGACG 00CTTGACG
TGGACGACG 00CTTGACG 00CTTGACG 00CTTGACG 00CTTGACG 00CTTGACG 00CTTGACG 00CTTGACG 00CTTGACG 00CTTGACG
581 THR*YIPH 1YTHRDLAR 2AGHSLRPR 3ICGLNHLN 4AFZOVALL 5POLYTHROR 6YSLHARPHS 7SLKHLKSL 8ALALAPHE 9LEUC*ALAOLN
PRO*ALAOLN 10ALAPHE 11TYL*YLEU 12TYL*YLEU 13TYL*YLEU 14TYL*YLEU 15TYL*YLEU 16TYL*YLEU 17TYL*YLEU 18TYL*YLEU
LEUC*OC 19TYL*YLEU 20TYL*YLEU 21TYL*YLEU 22TYL*YLEU 23TYL*YLEU 24TYL*YLEU 25TYL*YLEU 26TYL*YLEU 27TYL*YLEU

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48. After the attached sequence printout on page 65 of my notebook 22265 (ADE-19, p. 65), prior to March 17, 1997, I noted "The sequence confirms that this is a death domain-containing receptor. The receptor is clearly a new member of the TNF receptor family. It has cysteine-rich domains in the putative extracellular region, a transmembrane putative domain, and a cytoplasmic putative region that contains the death domain."

#### B. Work Conducted on My Behalf

49. After conducting my sequence analyses, and prior to March 17, 1997, I requested Mr. Scot Marsters to begin work to identify full-length clones from cDNA libraries using probes and primers designed from the ss.con1DD sequence. This cloning work is reflected in Example 1 of the '615 specification. (ADE-18, p. 58, l. 28 to p. 62, l. 14).

50. As Mr. Marsters continued working on the characterization of the Apo-2 receptor, he and Lakshmi Ramakrishnan developed a nomenclature scheme for the DNA and amino acid sequences that eventually were designated Apo-2 receptor.

51. A number of cDNA clones were isolated by Lakshmi Ramakrishnan from a cDNA library using the probes designed by Mr. Marsters.

52. A clone designated DD.2 was selected for further evaluation and was sequenced in its entirety by the Genentech sequencing facility. This work is also reported in Example 1 of

the '615 specification. (**ADE-18**, p. 58, l. 28 to p. 62, l. 14).

53. Concurrent with the sequencing work of DD2.1, Mr. Marsters transfected and overexpressed the DD2.1 clone in 293 cells to examine whether the protein encoded by the DD2.1 clone could potentially induce cell death in the 293 cells.

54. Dr. James Sheridan assisted Mr. Marsters with expression work and Dr. Sheridan confirmed that the DD2.1 clone killed the 293 cells. I personally reviewed the photographs of the cells as well as personally observed the cells under the microscope. This work is described in Example 4 of the '615 specification. (**ADE-18**, p. 64, l. 8 to p. 65, l. 13).

55. Mr. Marsters also worked with Maya Skubatch and began construction of fusion proteins using either the predicted extracellular domain of the DD.2.1 cDNA fused to DNA encoding an IgG Fc fragment, or the predicted extracellular domain of DD2.1 with a FLAG tag, which was used to help isolate the soluble protein. Mr. Pitti worked with the fusions as well. This work is reported in Example 2 of the '615 specification. (**ADE-18**, p. 62, l. 16 to p. 63, l. 6).

56. Mr. Marsters and Ms. Skubatch also conducted a variety of Northern blot analyses to identify expression of the mRNA encoded by the DD2.1 clone in multiple tissues. This work is reported in Example 7 of the '615 specification. (**ADE-18**, p. 67, ll. 11-35).

57. Mr. Pitti and Mr. Marsters conducted various binding studies using the Apo-2 ligand and the fusion proteins made by Mr. Marsters and Ms. Skubatch. Soon after conducting the binding studies, the nomenclature began to shift from DD.2.1 to "Apo-2" because it was observed that the Apo-2 ligand bound to the DD2.1 fusion protein. This work is reported in Example 5 of the '615 specification. (**ADE-18**, p. 65, ll. 15-28).

58. Mr. Marsters also conducted several assays demonstrating that the Apo-2 receptor activated the NFκB pathway. This work is reported in Example 6 of the '615 specification. (ADE-18, p. 65, l. 30 to p. 67, l. 9).

59. Mr. Marsters, Ms. Skubatch, and Dr. Sheridan conducted chromosome mapping experiments and determined that the gene encoding the Apo-2 receptor mapped to chromosome 8. This work is described in Example 8. (ADE-18, p. 68, ll. 1-15).

**C. My Work With Genentech Patent Counsel**

60. In the course of my research at Genentech, on various occasions I worked with a Genentech patent attorney named Diane Marschang.

61. In 1997, I began discussing my work relating to the Apo-2 receptor with Ms. Marschang. I do not presently recall the specific date on which I discussed or communicated my Apo-2 work to Ms. Marschang, but I believe it could have been as early as March or April of 1997.

62. The earliest documentary evidence of which I am presently aware reflecting my communication of Apo-2 related information to Ms. Marschang is dated May 5, 1997.

63. On or about May 5, 1997, I submitted to the Genentech Legal Department a Research and Development Information Release Application (RDIRA). (ADE-11). The Genentech Legal Dept. date stamp of May 5, 1997 is located on the upper right corner of the front page of the RDIRA.

64. The RDIRA was an internal approval form used by Genentech scientists prior to submitting a manuscript to a journal or other outside entity for publication.

65. In the "Title of Report" line in section 1 of the RDIRA, I entered the following:  
"A Second Cell Death Receptor for the Cytokine Apo-2 Ligand."

66. In the "Proposed date of submission" line in section 1 of the RDIRA, I entered the following: "May 5, 1997."

67. In the "Author submitting for release" line in section 1 of the RDIRA, , I entered the following: "Avi Ashkenazi."

68. In the "name of patent attorney/agent who should review this material" line in section 1 of the RDIRA, I entered the following: "Diane Marschang" and lists her telephone extension "x5416."

69. I attached a copy of my manuscript titled "A Second Cell Death Receptor for the Cytokine Apo-2 Ligand" to the RDIRA that I submitted to the Genentech Legal Department. The manuscript has been marked as **ADE-12**.

70. I recall that Ms. Marschang received a copy of the manuscript and that we discussed the work described therein in both the context of the submission of the manuscript for publication, as well as in the context of drafting a patent application directed to the Apo-2 receptor work.

71. I recall reviewing drafts of the '615 application before the '615 application was filed at the United States Patent and Trademark Office. A draft of the '615 application (**ADE-10**) contains the following entry at the top of page 1: "Draft # 1- May 5, 1997" which I believe was a notation entered by Ms. Marschang to reflect that she completed the first draft of the '615 application on May 5, 1997. Based on the length and content of the May 5, 1997, draft application, I believe that I began assisting Ms. Marschang with the drafting of the application before May 5, 1997, and probably in conjunction with my drafting of the Apo-2 manuscript.

72. **ADE-13** is a subsequent draft of the '615 application prepared by Ms. Marschang with my assistance. The following entry is at the top of page 1 of **ADE-13** "Draft #2 – May 13, 1997." I believe this notation reflects that Ms. Marschang completed a second draft of the '615 application on May 13, 1997, which is consistent with my recollection of more than one draft of the application prior to filing the '615 application at the United States Patent and Trademark Office.

**V. Conclusion**

73. I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of any patents issuing from the above-identified application.

Date: 4/20/07

Avi Ashkenazi  
Avi Ashkenazi, Ph.D.

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re application of:	)
CAMELLA W. ADAMS <i>et al.</i>	) Docket No.: 22338-00904/P1101R2D1
Application No. 10/052,798	) Examiner: Eileen B. O'Hara
Filed: November 2, 2001	) Group Art Unit: 1646
For: Apo-2 RECEPTOR	) <u>Declaration in Support of Request for</u>
	) <u>Declaration of Interference</u>
	) <u>Under 37 C.F.R. § 41.202</u>
	) <u><b>Expedited Handling Requested</b></u>
	)
	)

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COMMISSIONER FOR PATENTS  
P.O. Box 1450  
Alexandria, VA 22313-1450

**DECLARATION OF ROBERT PITTI**

**ADE- 4**  
USSN 10/052,798

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## **DECLARATION OF ROBERT PITTI**

I, Robert Pitti, declare and state as follows:

### **I. Introduction and Background**

1. I am a citizen of the United States and presently reside in El Cerrito, California.
2. I received my Bachelor's of Arts degree in English from San Diego State University and my Bachelor's of Science degree in Biological Sciences from the University of California, Davis.
3. I have been employed by Genentech Inc., South San Francisco, CA ("Genentech") since June 1984.
4. From 1995, until the present, I have worked as a Senior Research Associate in the Department of Molecular Oncology in Dr. Avi Ashkenazi's lab.
5. In this declaration, I refer to pages in my laboratory notebooks submitted as **ADE-20** and **ADE-21**.
6. The handwriting on the cited notebook pages is my handwriting, unless otherwise noted. I typically signed and dated my notebook pages on the days that I began the experiments.
7. The descriptions I provide in this declaration include images from portions of my notebook pages. The notebook pages are reproduced in their entirety in the exhibits submitted herewith. The images embedded in this declaration are accurate reproductions of the notebook pages and are intended to help guide the reader in considering my notebook entries.
8. I understand that Genentech intends to file this declaration at the United States Patent and Trademark Office to establish activities that occurred prior to March 17, 1997, and up to May 15, 1997. I understand that March 17, 1997, is a date by which another company filed a patent application relating to the Apo-2 receptor (also known as DR5) and that May 15, 1997, is

the date on which Genentech filed a patent application relating to the Apo-2 receptor. Dates preceding March 17, 1997 on documents cited in this declaration have been redacted.

9. In this declaration, I provide the following:

- a. An overview of the personnel with whom I interacted at Genentech during my employment during the years 1996-1998;
- b. An overview of the types of experiments that I conducted while working for Dr. Ashkenazi; and
- c. A detailed description of my activities relating to the work I conducted using the Apo-2 receptor.

## **II. Overview of the Personnel With Whom I Interacted At Genentech**

10. In the course of my work during 1996-1998, I routinely interacted with several Genentech scientists including: Dr. Ashkenazi, Scot Marsters, Maya Skubatch, and Dr. James Sheridan. I routinely discussed my work and experimental results with these scientists during our interactions in the close proximity of the Ashkenazi lab. These scientists, including Dr. Ashkenazi, would discuss their results and objectives with me as well.

11. Prior to March 17, 1997, I understood that Dr. Ashkenazi was searching for receptor molecules in the Tumor Necrosis Factor (TNF) family of receptors. I understood that Dr. Ashkenazi was searching for novel receptor sequences based on structural similarities to previously identified receptors in the TNF family (*e.g.*, Apo-3 and Fas), including sequences for 1) cysteine-rich regions in the extracellular domain, 2) a transmembrane domain; and 3) an intracellular region containing a death domain.

12. I recall that prior to March 17, 1997, Dr. Ashkenazi searched an Incyte sequence database and identified DNA sequences that he considered to be similar to the sequence of the Apo-3 receptor.

13. Prior to March 17, 1997, I clearly understood Dr. Ashkenazi's objectives regarding the newly identified receptor molecules. I worked in the Ashkenazi laboratory and Dr. Ashkenazi and I often discussed the progress of my research and his research objectives. At that time, I understood that upon identifying a novel receptor sequence, Dr. Ashkenazi planned to conduct cloning and expression experiments as well as to prepare a fusion protein (Apo-2-IgG (immunoglobulin G)) to use in binding studies. Additionally, I understood that Dr. Ashkenazi planned to initiate experiments to prepare antibodies against the receptor, including the preparation of agonist antibodies that would specifically bind to the extracellular domain (amino acids 54 to 182) of the newly identified receptor, and that would induce apoptosis in a DR5-expressing cell.

14. Specifically, prior to March 17, 1997, I understood that Dr. Ashkenazi had contemplated a method of inducing apoptosis in a DR5-expressing cell which involved exposing the cell to an effective amount of an Apo-2 agonist monoclonal antibody which (a) binds to a soluble extracellular domain sequence (amino acids 54 to 182) of an Apo-2 polypeptide and (b) induces apoptosis in a DR5-expressing cell.

15. Once Dr. Ashkenazi identified sequences in the Incyte database, I recall that he asked Scot Marsters to design PCR primers and DNA probes based on the sequences Dr. Ashkenazi identified. Mr. Marsters designed the primers, designated them "DD" primers and a "DD" probe, and asked a research associate collaborating with the Ashkenazi laboratory, Lakshmi Ramakrishnan, to screen various cDNA libraries and isolate clones having structural similarities to the sequences Dr. Ashkenazi identified.

16. I recall that Ms. Ramakrishnan isolated a clone designated “DD2”. The “DD2” designation is a reflection of Scot Marsters’ probe designation (“DD”) and number “2” was the second positive clone (out of four) identified by Ms. Ramakrishnan.

### **III. Overview of My Work Involving the Apo-2 Receptor**

17. My own laboratory work involved working with DNA constructs having DD2 DNA inserts. Scot Marsters was the person who provided me with these DNA constructs. I recall that the “DD2” DNA was determined to encode the molecule later designated the Apo-2 receptor.

18. My laboratory work included creating and characterizing the expression of different DNA constructs containing the DD2 DNA. Specifically, my work included making and/or assaying DD2-IgG (antibody) immunoadhesion fusion constructs; making and/or assaying DD2-Flag fusion constructs; and making and/or assaying DD2-GST (glutathione-s-transferase) fusion constructs.

19. I also conducted binding assays to evaluate the binding characteristics of the receptor encoded by the DD2 DNA with a molecule called Apo-2 Ligand (“Apo-2-L”). My experiments reflect that Apo-2-L bound with specificity to the receptor encoded by the DD2 DNA. I conducted traditional competition-based binding experiments, as well as experiments using a Biacore chip having the receptor linked thereto which was then incubated with Apo-2-L.

### **IV. Detailed Description of My Laboratory Notebook Entries**

#### **A. Relevant Entries From Notebook Number 26610 (ADE-20)**

##### **1. Page 68, Notebook 26610**

20. I recorded and conducted the activities described on page 68 prior to March 17, 1997, as reflected by the date I recorded on that page next to my signature.

21. At the top of page 68, I recorded the title of the experiments as “Gel Purification of GST Fusion PCR Products” reflecting that I gel-purified GST Fusion PCR products. I would

likely have received the GST fusion PCR products from Scot Marsters. At the time of these experiments, I would often be asked to help complete an experiment using materials generated by others in the lab such as Scot Marsters.

22. A protein fused to a GST moiety allows for purification of the fusion protein using commercially available products that bind to the GST molecule. Therefore, the purpose of the experiment was to generate purified DD2-GST fusion proteins to obtain purified DD2 protein.

23. At the top of page 68, I attached a photograph of a gel containing PCR reaction products. I made a notation at the top of the image of the gel which reads “GST 15 cycles DD2” reflecting that the PCR products run on the gel encoded GST-DD2 fusion constructs.

24. The entries “L<sub>10</sub>” and “L” and “S<sub>10</sub>” and “S” reflect that there were long (“L”) and short (“S”) forms of the GST-DD2 fusion construct. The difference in long and short forms related to the length of linker sequence contained between the “GST” and “DD2” portions of the fusion construct. Multiple fusion lengths were often used to ensure that the constructs would be effectively expressed in an expression system.

25. I purified the PCR fragments from the gel and ligated the PCR fragments into plasmid vectors. I described my continued activities using the plasmids I made on page 69.

## **2. Pages 69-70, Notebook 26610**

26. I recorded and conducted the activities described on pages 69 and 70 on March 17, 1997, as reflected by the dates I recorded on those pages next to my signature.

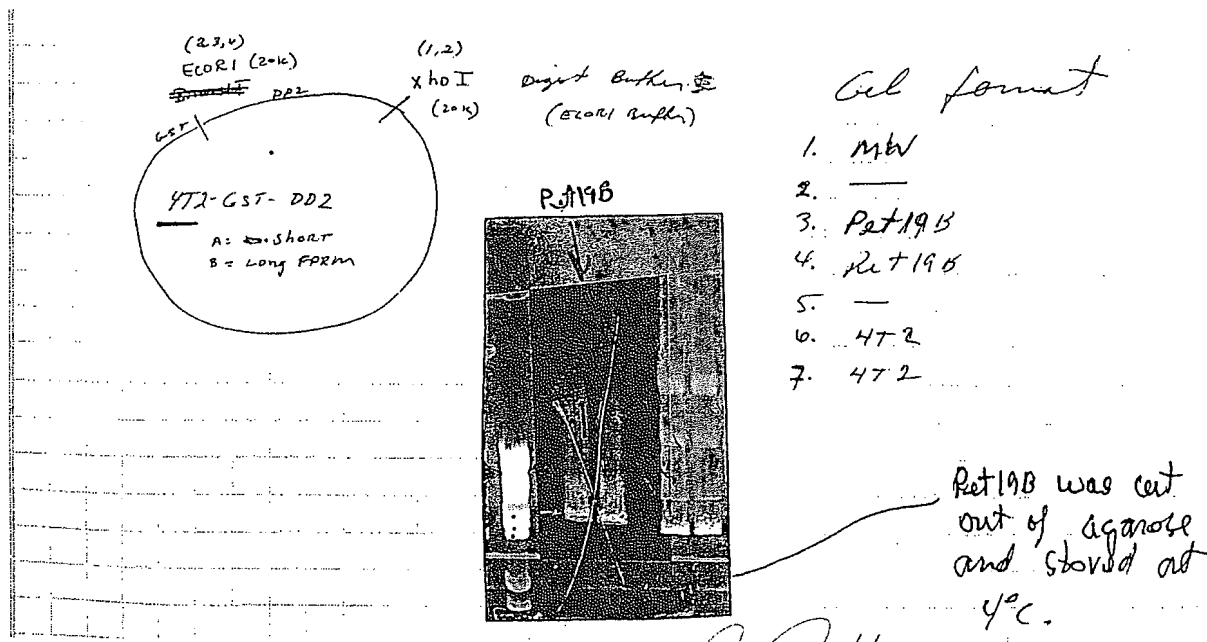
27. At the top of page 69, I recorded the title of the experiments as “Plasmid digests” reflecting that I conducted restriction digests of plasmids. Based on my notebook entries, the plasmids I digested were from the experiment I described on page 68.

28. I recorded the reaction conditions that I followed for the plasmid digests at the middle of page 69 toward the right side. The entries under “X10µl 4T2” correspond to the reagents and amounts I used to digest the GST-DD2 fusion construct. I digested the construct with the restriction enzymes EcoR1 and XhoI which were expected to yield a band on a gel containing the DD2 insert because the DD2 insert was surrounded by the EcoR1 and XhoI restriction sites.

29. The image below is of entries that I made on page 69. The circular diagram is a schematic of the GST-DD2 fusion construct and highlights the predicted digestion sites from EcoR1 and XhoI and the location of the DD2 and GST inserts.

30. I also listed samples 1-7 under the “Gel format” entry on page 69. Lanes 6 and 7 contain the digested products from the “4T2” construct, which is the same as the schematic I drew, and contained the DD2 and GST inserts.

31. The gel I attached at the bottom of page 69 is a photograph of an analytical gel which I used to confirm that the digested products were present in the samples I prepared. The last two lanes of the gel contained the GST-DD2 fusion digests and demonstrated the expected banding pattern.



32. At the top of page 70, I wrote "Acrylamide" and "PCR & Purification" reflecting that I ran additional aliquots of plasmid digests (described on page 69) on a preparative acrylamide gel. I ran 5 microliter aliquots from the same digests I described on page 69 on a 6% acrylamide gel and purified ("electro-eluted") the bands corresponding to the GST-DD2 fusion products. This process yielded purified DNA products encoding the GST-DD2 fusion insert.

33. I attached a photograph of the acrylamide gel at the top of page 70.

34. I made the following entries at the middle of page 70:

The L10 and S10's were cloned into pGEMT  
(The L+S were as well, but these were disregarded)  
The pGEM clones were PCR selected & then EcoRI/XhoI  
digestion, 5 of each. Of these 4 long and  
3 short were submitted for sequence.

35. My entries reflect that the purified GST-DD2 samples corresponding to "L<sub>10</sub> and S<sub>10</sub>" from the acrylamide gel, were cloned into a "pGEMT" vector. A pGEMT vector was a readily available vector used for making DNA constructs. The newly ligated clones were then

“PCR selected” and then digested with the restriction enzymes EcoR1 and XhoI. The image of the gel from the restriction digest is taped to the bottom of page 70. The GST long samples were labeled 2, 3, 4, 5, and 6 whereas the GST short samples were labeled 50, 51, 52, 53, and 54.

36. I noted that “4 long and 3 short were submitted for sequence” reflecting that four clones having the long form of the construct (samples 2-5) and 2 clones (samples 52-53) having the short form of the construct were submitted to the sequencing facility.

### 3. Page 72, Notebook 26610

37. I recorded and conducted the activities described on page 72 on March 18, 1997 as reflected by the date I recorded on that page next to my signature.

38. At the top of page 72, I recorded the title of the experiment “PCR Frags for GST-DD2 PGEM Ligation” reflecting that I conducted additional PCR experiments to amplify DD2-GST inserts and then ligated the additional GST-DD2 PCR fragments into pGEM vectors. This experiment is similar to the experiments I described on page 70 where I cloned the digested DD2-GST fusions into pGEM.

39. At the middle of page 72, I made the following entries reflecting the reagents I used in the ligation reaction. I listed the GST-DD2 PCR fragments beside each of the A, B, C and D entries. “L<sub>10</sub>” reflects that the long form of the fusion was used in PCR reactions and the PCR reaction ran for 10 cycles. “L<sub>15</sub>” reflects that the long form of the fusion was used in PCR reactions and the PCR reaction ran for 15 cycles. “S<sub>10</sub>” reflects that the short form of the fusion was used in PCR reactions and the PCR reaction ran for 10 cycles. “S<sub>15</sub>” reflects that the short form of the fusion was used in PCR reactions and the PCR reaction ran for 15 cycles. I then listed the reagents and conditions used for the vector ligation experiment under the A, B, C, and D entries.



A. L10 } long 10 + 15 cycle PCR  
 B. L15 }  
 C. S10 } short 10 + 15 cycle PCR  
 D. S15 }  
 low recovery

T4 Buffer	1ul	✓
VECTOR	1ul	✓
PCR	5ul	✓
Ligase	1ul	
dH <sub>2</sub> O	2ul	✓
	10ul	TOTAL

3 hours @ 15°C

40. Following the ligations, I then had multiple plasmid preparations containing the DD2-GST fusion construct cloned into pGEM vectors.

#### 4. Pages 73-75, Notebook 26610

41. I recorded and conducted the activities described on pages 73-75 on March 19, 1997, as reflected by the dates I recorded on those pages next to my signature. The experiments on these pages involved transfecting 293 cells with DD2 DNA constructs, lysing the cells following transfection, and measuring the protein content of the cell lysates.

42. At the top of page 73, I recorded the title of the experiment as "293 TFX and Cell Lysis" reflecting that I conducted transfection experiments of 293 cells and lysed the cells to isolate protein expressed from the transfected cells.

43. At the middle of page 73, I made the following entry:

6cm plates of confluent 293's were transfected in Calcium Phosphate procedure until the following DNA.

plate	Receptor	Crma	PRK5
1	10 DD2	0	
2	10 TNFR1	0	
3	10 APO3	0	
4	10 DD2	10	
5	10 TNFR1	10	
6	10 APO3	10	
7	10 PRK5	0	
8	10 PRK5	10	

3 plates were transfected for each and incubated O/N at 37°C.

44. The entries I made in the image above reflect that I transfected confluent 293 cells in 6cm plates with DD2 DNA using a calcium phosphate procedure. I listed the plate numbers (1-8) along the left side of page 73 and the corresponding DNA constructs I used to transfect the cells. I transfected the cells on plates 1 and 4 with DD2 constructs. I noted that I co-transfected the cells with a construct containing the gene encoding CRMA (a caspase inhibitor), on plate 4 as reflected by the "10" under the "Crma" column. Caspase inhibitors would block apoptosis induced by expression of the DD2 constructs and were added to prevent unwanted apoptosis in those cells.

45. I wrote "3 Plates were transfected for each and incubated O/N at 37°C" reflecting that I performed the transfections in triplicate (3 plates were transfected for each plate number shown above) and incubated the transfected cells overnight (O/N) at 37°C.

46. At the middle of page 73, I made the following entry:

Lysis

1. Cells were spun and washed with PBS
2. Cells were lysed with
  - 1% NP-40 10mM TRIS
  - 150mM NaCl
  - PMSE, Aprotinin etc.

for 15 minutes.

47. These entries reflect that I centrifuged the transfected 293 cells and then lysed the cells using a cell lysis buffer for fifteen minutes.

48. Following lysis of the cells, I assayed the protein concentration of the cell lysates. These activities are described on page 74.

49. The activities I described on page 74 were directed to measuring the protein content in the 293 cells I transfected as described on page 73. I assayed the optical density of the samples in a spectrophotometric reader at 562 nanometers ("OD 562 nm") and recorded the readouts at the middle of page 74 as reflected in the image of page 74 below.

STD BSA Curve				Samples OD 562 (1:20)		
1	1000 µg/mL	→	OD 562 nm			
2	500	1.388	780	1	(327) .518	$\times 20 = 6.54 \text{ mg/mL}$
3	250		451	1	(195) .314	$\times 20 = 3.90 \text{ mg/mL}$
4	125		244	3	(324) .513	$\times 20 = 6.48 \text{ mg/mL}$
5	62.5		140	7	(344) .544	$\times 20 = 6.88 \text{ mg/mL}$
6	31.25		080	5 (377) (294)	.594	$\times 20 = 7.54 \text{ mg/mL}$
7	15.625		046	6	(294) .467	$\times 20 = 5.88 \text{ mg/mL}$
8	0			7	(302) .479	$\times 20 = 6.04 \text{ mg/mL}$
				8	(258) .412	$\times 20 = 5.2 \text{ mg/mL}$

50. The numbers I recorded on the left side is a standard concentration curve used to determine approximate protein concentration in a sample. I wrote "STD BSA Curve" at the top of the samples listed 1-8 on the left side of the entry above. "BSA" is an abbreviation for bovine serum albumin which is used as a protein standard. Next to the samples numbered 1-8 is the

concentration (in ug/ml) of the serial dilutions I made of the BSA standards I used to generate the standard curve. To the right of the concentration entries is the optical density reading of the samples measured at 562 nm in the spectrophotometric device.

51. The right side of the table shows the sample number and the optical density (OD) of each sample measured at 562 nm. Based on the optical densities obtained from the serial dilutions in the standard curve, having known concentrations of BSA, I was able to predict the protein concentration in the transfected 293 cell lysates. The entries on the right side of the page 74 reflect the predicted protein concentration in the cell lysates obtained from the transfected 293 cells. Samples 1 and 4 had predicted protein concentrations of 6.54mg/ml and 6.88 mg/ml, respectively.

**5. Page 76-78, Notebook 26610**

52. I recorded and conducted the activities described on page 76 on March 24, 1997 as reflected by the date I recorded on that page next to my signature. I recorded and conducted the activities described on pages 77 and 78 on March 26, 1997 as reflected by the date I recorded on those pages next to my signature.

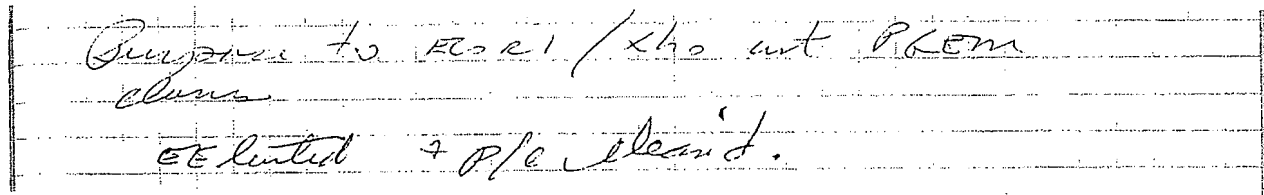
53. The purposes of the experiments described on pages 76-78 were to: 1) create a vector (pGEX) to receive a DD2-GST insert; 2) digest existing vector to obtain the DD2-GST insert; and 3) ligate the DD2-GST insert into the pGEX vector.

54. At the top of page 76, I recorded the title of this experiment as “4T2 PGEX Plasmid Prep” reflecting that I prepared “4T2 PGEX” plasmids for use in later ligation experiments. I recorded the experimental conditions at the middle of page 76 and included a photograph of a gel I ran to confirm that the PGEX had been properly digested with the restriction enzymes EcoR1 and XhoI.

55. Based on my notebook entries on pages 77 and 78, I ligated a DD2-GST insert into the plasmids I prepared as described on page 76.

56. At the top of page 77, I recorded the title of the experiments as “R1-XhoICuts GST-DD2 L&S PGEM” reflecting that I conducted restriction digests of PGEM ligated vectors containing an EcoR1-XhoI GST-DD2 fragment. The PGEM vectors I digested were from samples “5” and “54” that I described on pages 70 and 72 of my notebook..

57. At the top of page 77, I made the following entry:

A photograph of a handwritten entry on a lined notebook page. The text is written in cursive ink. The first line reads "Purpose to EcoR1/XhoI cut PGEM clones." and the second line reads "electeluted + p/e clean it." The handwriting is somewhat slanted and the ink is dark.

Purpose to EcoR1/XhoI cut PGEM clones.  
electeluted + p/e clean it.

58. These entries reflect that the purpose of this experiment was to digest pGEM vectors with the restriction enzymes EcoR1 and XhoI to obtain a desired restriction fragment, which in this experiment, was a GST-DD2 fragment. The entries also reflect that the fragments were electroeluted from the gel and purified using a phenol/ethanol extraction (“p/e”).

59. I attached a photograph of the gel on which I ran the digest products to page 77. I wrote “5” and “54” at the top of two of the lanes on the photograph of the gel reflecting that I digested clones “5” and “54” (previously identified on the gel photograph on page 72) and ran aliquots from the digests on a gel. I labeled the photograph of the gel “R1/Xho cut from PGEM cl.5 + 54” confirming that aliquots from clones 5 and 54 were the run on the gel.

60. At the top of page 78, I wrote “Rapid Ligation” reflecting that I would conduct a ligation experiment to ligate the fragments obtained from samples 5 and 54 described on page 77 into a vector.

61. Under the "Procedure" entry I made on page 78, I listed the procedure I followed and reagents I used to conduct the rapid ligation experiment. The vectors I used were the pGEX vectors I made on page 76 and the inserts I used were from the DD2-GST inserts I prepared on page 77.

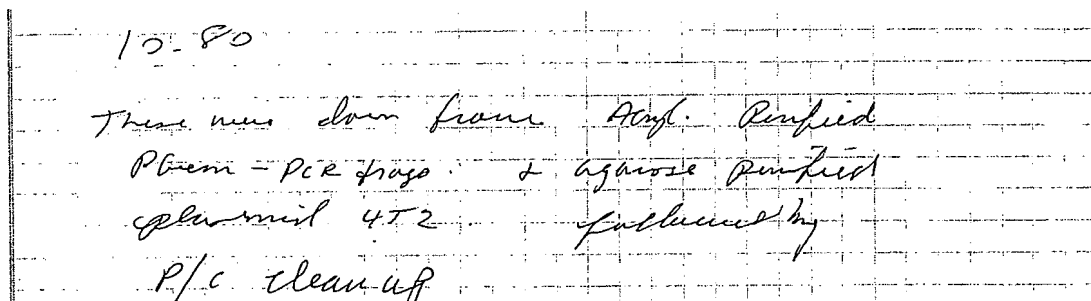
62. Once I made the new DD2-GST-pGEX constructions, these constructions were now readily available for use in subsequent experiments.

**6. Page 80, Notebook 26610**

63. I recorded and conducted the activities described on page 80 on March 31, 1997 as reflected by the date I recorded on that page next to my signature.

64. At the top of page 80, I recorded the title of the experiment "GST Ligation and Transformation." The entries I made on page 80 were "housekeeping" entries and reflect the status of the constructs that I worked on from the previous week's work.

65. At the middle of page 80, I made the following entries as reflected in the image below.



10-80  
These were done from Aug. 1997.  
Plasmid - PCR frag. & agarose purified  
followed by  
P/c clean-up

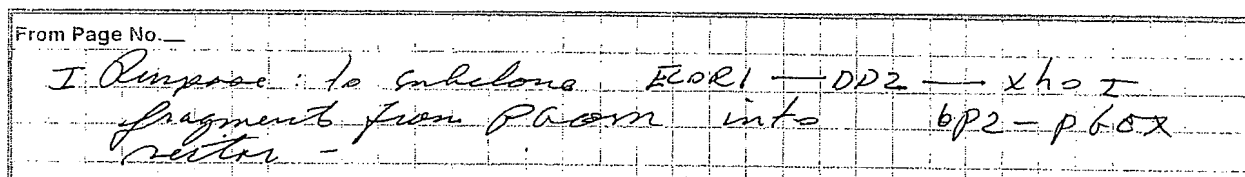
66. The entry "10-80" corresponds to the last two digits from my notebook 26610 in which I made this entry, and the page number. I would typically use this nomenclature when labeling tubes containing preparations that I made in the laboratory. The entry on page 80 served as an inventory for materials I labeled "10-80" and put into storage. The entry reflects that I

prepared acrylamide purified pGEM-PCR fragments and the agarose purified 4T2-pGEX vectors, which corresponds to the work I conducted during the previous week.

7. Page 83-85, Notebook 26610

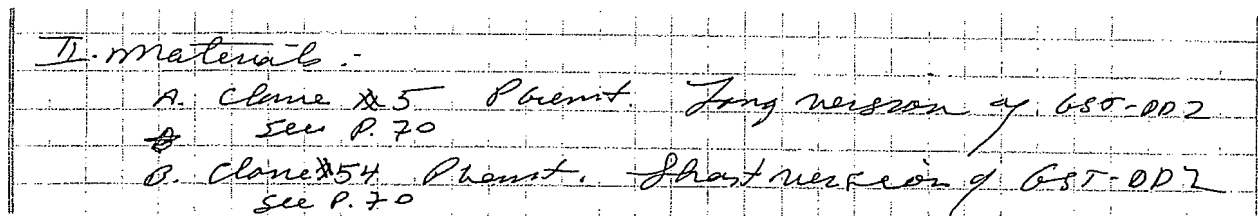
67. I recorded and conducted the activities described on pages 83-85 on April 3 and 4, 1997 as reflected by the dates I recorded on those pages next to my signature. The experiments described on pages 83-85 involve cloning experiments to place the DD2-GST fusion inserts into another type of pGEX vector.

68. At the top of page 83, I recorded the title of this experiment "4T2-pGEX DD2 Clones" and "6P2-pGEX DD2 Clones" and made several notebook entries relating to the experiments as reflected in the image of a portion of page 83 below.



69. As reflected in the image above, the purpose of this experiment was to subclone EcoRI-DD2-XhoI fragments from PGEM into a 6P2-pGEX vector. I note that I also cloned DD2-GST fragments into additional 4T2-pGEX vectors in this experiment. pGEX is an *E. coli* plasmid vector and pGEX-6P vectors permit site-specific cleavage and simultaneous purification on glutathione sepharose. The 6P2-pGEX vector was different from the 4T2-pGEX vectors I prepared earlier.

70. At the middle of page 83, I made additional entries as reflected in the image of a portion of page 83 below:



71. The entries I made on page 83 in the image above reflect the materials I used in the subcloning experiments. I used clone numbers 5 and 54, which I previously cloned into separate pGEMT vectors as I first described on page 70.

72. Under the “Procedure” entry at the middle of page 83, I listed the procedures that I followed to digest the 6P2-PGEX and 4T2-PGEX vectors in preparation of the cloning experiment and the specific reagents that I used. I digested both the 6P2-pGEX and 4T2-pGEX vectors with EcoR1 and XhoI and ran the digested products on a 1% agarose gel for two hours at 37°C. I “cleaned up” the digest products from the gel I ran with a Qiaquick purification system, which is a commercially available gel purification kit.

73. Following the digests and purification, I obtained purified “host” vectors 6P2-pGEX and 4T2-pGEX to receive DD2-GST DNA inserts which would be digested using the same restriction enzymes thereby yielding an insert that would readily ligate into the 6P2-pGEX and 4T2-pGEX vectors.

74. The next step I conducted was to digest the “donor” pGEMT clones (numbered 5 and 54) to obtain the DD2-GST insert for the pGEX vectors.

75. At the top of page 84, I made the following entry as reflected in the image below:



From Page No. \_\_\_\_\_

2. Phem-T clones were (#5 & #54) were  
cut as above in next section and  
run out on 2% mini-agarose gel  
at 100V & cleaned up with QIAquick  
system

76. My entries in the image of page 84 above reflect that I digested pGEM-T clones (#5 and #54) as described on page 83 for the pGEX vectors (*i.e.*, using the same restriction enzymes EcoR1 and XhoI), ran the digests on a gel, and purified the fragments using a Qiaquick gel purification system.

77. At the top of page 84, I made the following entry:

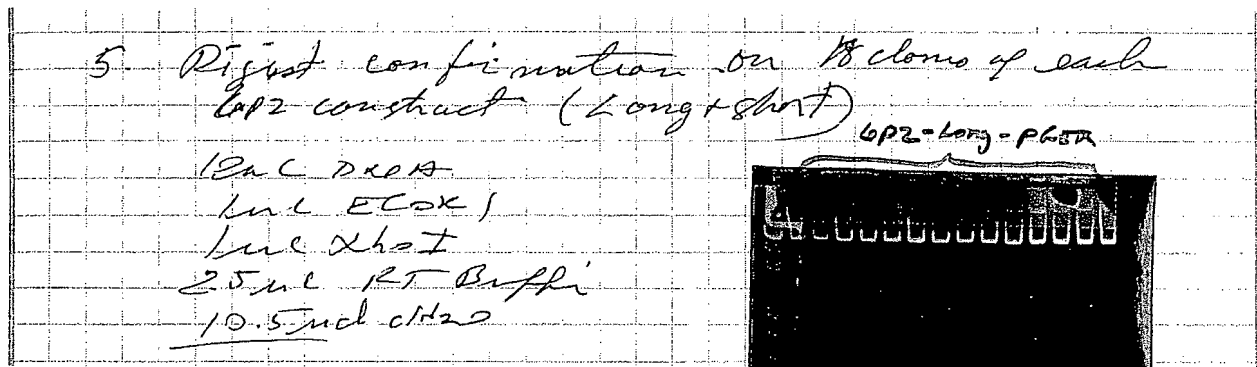
3. Ligation was done o/n with  
T4 ligase at 16°C  
(NBB 20266)

1 mL vector  
5 mL insert  
1 mL T4 Ligase  
5 mL Ligase Buffer  
38 mL dH<sub>2</sub>O  
50 mL Total

78. The entry I made at number "3." on page 84 reflects that I ligated overnight the purified DD2-GST fragments, obtained from the pGEM digests, into the previously prepared 4T2-pGEX and 6P2-pGEX vectors. I listed the reagents I used and the reaction conditions I followed in the ligation experiment at the middle of page 84 under the number "3." entry.

79. At the bottom of page 84, and top of page 85, at entry "4.", I noted that I transformed the newly ligated vectors into DH10B (E.coli) cells for one hour and plated the transformants on "LB" agar plates supplemented with the selection antibiotic carbenicillin. Transformants that survived on the selection media contained the vector which carried the gene conferring antibiotic resistance to transformed bacterial cells. I recorded the transformation conditions I followed at the bottom of page 84.

80. Following the transformation, I allowed the bacterial cells to incubate overnight at 37° Celsius. ("O/N at 37°C"). Following incubation of the cells overnight, I identified positive clones for the 6P2-pGEX constructs and conducted a confirmation digestion on eighteen clones of each of the 6P2-pGEX constructs I made. This experiment allowed me to confirm that the clones contained the expected DD2-GST inserts. These activities and the reagents I used in the digest are reflected in the image of the portion of page 85 at item "5." set forth below.



81. I note that although page 85 is dated April 3, 1997, I conducted the confirmatory digest described at item "5." on page 85 on April 4, 1997 because I allowed the transformed bacterial cells to incubate overnight starting on April 3, 1997.

82. I attached photographs of the three gels from the confirmatory digests I conducted to page 85 and wrote the following conclusion at the bottom of the page: "Conclusion: Subcloning was successful. All clones positive." This entry reflects that I successfully cloned

the DD2-GST fusion protein into the 6P2-pGEX vector. These newly constructed pGEX vectors containing the DD2-GST fusion construct were now available for use in experiments.

83. From April 7-9, 1997, I attended a scientific conference and did not conduct any laboratory activities on these days.

**8. Pages 86-90, Notebook 26610**

84. I recorded and conducted the activities described on pages 86-90 on April 10 and 11, 1997 as reflected by the dates I recorded on those pages next to my signature.

85. The purpose of the experiments I recorded on pages 86-90 was to confirm that the DD2-GST expression vectors I constructed as described on pages 83-85, were functional. I transformed *E. coli* with the pGEX plasmids I prepared containing the DD2-GST fusion. I then assayed the amount of GST present in the supernatant of the bacterial cells after sepharose purification, using a commercially available assay kit (CDNB assay). The CDNB assay is a colorimetric assay measuring the conjugation of CDNB to reduced glutathione. The protocols I followed, and the results I obtained, are set forth on pages 86-90.

86. I taped a typed protocol titled "Expression of GST fusions in *E. coli*" to page 86 which lists the steps I followed to 1) transform *E. coli* cells (under "Procedure; A. Growth and Extraction"); and 2) purify the GST fusion proteins expressed by the bacteria using GST-Sepharose resin (under "B. Purification").

87. I noted at the top of page 86 that "Expression: 0.659 when IPTG was added A<sub>600</sub>" reflecting that the bacteria were grown to a density having an absorbance of 0.659 at the time Isopropyl-β-D-thiogalactopyranosid (IPTG) was added to the cells. This is consistent with entries 1-3 in the protocol I taped to page 86 and reflected in the image below.

### Expression of GST fusions in E.Coli

#### Materials:

Ice Cold PBS  
2YT/Carb  
IPTG (200 mM) Genentech Stock. This 200x off the shelf.  
20% Triton X 100. Prepare from 100% stock at least 1 hour ahead

#### Procedure

##### A. Growth and Extraction

1. Inoculate 100 mL of 2YT with a colony of E.coli cells containing pGEX plasmid and incubate overnight at 37 degrees C.
2. Warm 2YT/carb media to 37 degrees C and dilute the stock E Coli 1:100 and grow to A600 of .5-2.
3. Add IPTG 1:2000 and incubate an additional 2-6 hours
4. Transfer to 500 ml bottles and centrifuge for 10 minutes at 7,700 g. (GS-3 at 7000 rpm) at 4 degrees C.
5. Drain the pellet and place on ice immediately.
6. Resuspend the pellet in ice cold PBS, 50 mL per Liter of original culture volume. Transfer to 50 mL conicals.
7. Adjust volume of suspension to about 40 ml per tube. Sonicate on ice twice for 25 seconds on #5 using the large probe. Save an aliquot of the sonicate for GST CDB assay at this point. Assay bacteria from pre-sonicate along side sonicate to see that the protein has been released.
8. Add 20 % Triton X-100 to a final concentration of 1% (2.6 mL per 50 mL of sonicate). Mix gently for 30 minutes at 4 degrees C.
9. Transfer to Oakridge tubes and spin at 12,000 g for 10 minutes at 4 degrees C.
10. Transfer the supernatant to 50 ml conicals. This can be frozen at this step.

##### B. Purification

1. Preparation of 50 % slurry ( will need: 1 ml of GST-Sepharose 50% slurry for each Liter of original culture volume).
  - A. Gently shake the bottle of gel to resuspend.
  - B. Pipette 1.33 ml of slurry for each liter of original culture volume.
  - C. Sediment the gel by centrifuging at 500 g for 5 minutes.
  - D. Wash each 1.33 mL of slurry with 10 mL of cold PBS and repeat centrifugation.
  - E. For each 1.33 mL of slurry resuspend with 1 mL of PBS to yield a final concentration of 50% slurry.
2. Add slurry to the supernatant (1 mL of slurry for each liter of original culture volume) and incubate with gentle agitation at RT for 30 minutes.
3. Centrifuge at 500 X for 5 minutes to sediment the gel. Remove the supernatant and save for assay.
4. Carefully load gel onto a column. Do not trap air bubbles
5. Gravity wash 3x 10 mL with PBS per ml of resin. Check flow-through with Bradford (Note BCA and Lowry cannot be done here after because of the presence of glutathione).
6. Elute with 1 mL of Glutathione Elution Buffer per 2 ml of 50% slurry. Add directly to the column and mix with resin. Let stand at RT for 10 minutes then drip through into clean eppendorf tubes.
7. Repeat elution at least twice. Peak elution may not be the first one, so these repeated elutions may be critical. Place fractions on ice immediately and assay be CDB and Bradford.

*See Page 89-90  
for commercial  
procedure*

88. I then followed the remainder of the steps recited in the protocol I taped to page 86 to grow, extract, and purify the GST proteins obtained from the bacteria I inoculated with the vectors containing the DD2-GST fusions.

89. At the top of page 87, I recorded the title of the experiment: "GST-DD2 Expression & Purification CDB Assay." The CDB assay is a commercially available assay kit used to evaluate the GST content in samples and I taped the protocol I followed to page 89. The CDB reagent reacts with the GST protein and causes a shift in the absorbance of samples containing GST protein.

90. I recorded the absorbance values on page 87 for samples containing both the GST-DD2 short and long fusion proteins. The data is set forth in the image from page 87 below. The first column on the left under "secs" reflects the number of seconds at which I assayed the

sample tubes containing the GST fusion proteins. The second column under “GST DD2-short” reflects the absorbance values at 340 nanometers measured at each time point for the GST-DD2 short sample. The third column under the “Long” entry reflects the absorbance values at 340 nanometers measured at each time point for the GST-DD2 long sample.

91. I taped a plot of the time course (in seconds) of the CDNB reaction (x –axis), versus the absorbance of the sample (y axis) to the right side of page 87. This plot, and the results of the experiment, reflects that the DD2-GST samples I prepared contained the GST fusion protein, therefore the DD2-GST expression constructs I made were functional.

92. Following confirmation of the presence of the GST fusion proteins in the samples using the CDNB assay I purified the GST fusion proteins using the protocol I taped to page 89.

93. At the top of page 89, I recorded the title of the experiment as “GST-DD2 Expression & Purification CDNB Assay.” In addition to the CDNB assay protocol, I attached the protocols I followed to 1) prepare glutathione sepharose beads (bottom of page 89); and 2) purify the DD2-GST fusion proteins (page 90) I prepared.

94. I followed the sepharose preparation protocol I taped to page 89 to prepare the slurry used to isolate the GST fusion proteins. The GST portion of the fusion proteins would bind to the sepharose resin and allow for purification of GST proteins by passing the bacterial samples over the sepharose preparation and conducting a series of washes of the mixture.

95. I followed the purification protocol that I taped to page 90 to purify the GST fusion proteins from the bacterial samples. I purified pooled “long” and “short” GST proteins into four samples for the short and long GST fusions (labeled S<sub>1</sub> through S<sub>4</sub> for “short” and L<sub>1</sub> through L<sub>4</sub> for long). I retained the samples I eluted from the sepharose column and planned to

conduct a protein detection assay (*e.g.*, Biorad) and run aliquots of the samples on a gel and subject the samples to silver staining to confirm the presence of the DD2-GST fusions

96. Following obtaining the four elution fractions from the DD2-GST purification, I conducted an assay to measure the total protein content of the supernatants obtained from the bacterial cells I transformed with the DD2-GST fusions. I recorded these activities on page 88 and recorded the title of the experiment as “Biorad on GST Material.”

97. “Biorad” is a designation for a protein assay kit available from a company called Biorad. Generally, the Biorad assay is a colorimetric assay wherein a sample is placed into a spectrophotometer and the light absorbance of the sample is measured. The greater the absorbance of a sample at a given wavelength, the greater the protein concentration in that sample. Protein standards (*e.g.*, BSA) are used to provide absorbances for known protein concentrations. The protein concentration in samples having an unknown protein content can then be predicted using the protein standards.

98. On page 88, I made the entries set forth in the image below.

BSA mg/mL		Abs 595	value	
0		0		
15		0.37		
31.25		0.59		
62.5		1.02		
125		1.86		
250		3.14		
500		6.32		
1000		8.44		

$$y = m_0 + m_1 * x$$

$$m_0 = 0.0292$$

$$m_1 = 0.0104529$$
  

				mg/mL
S <sub>1</sub>	1:10	.090	$\frac{y - .0292}{.001045} = x$	58.2
S <sub>2</sub>	1:10	.171		136.0
S <sub>3</sub>	1:10	.140		106
S <sub>4</sub>	1:10	.085		53.4
				$\Sigma =$

L <sub>1</sub>	1:10	.218		180.7
L <sub>2</sub>	1:10	.258		218.9
L <sub>3</sub>	1:10	.189		153
L <sub>4</sub>	1:10	.087		55.3
				$\Sigma =$

Total yield		total	
S <sub>1</sub>	.582	x .75	.4365
S <sub>2</sub>	1.360	"	1.020
S <sub>3</sub>	1060	"	.795
S <sub>4</sub>	.534	"	.4005
		$\Sigma =$	2.65
L <sub>1</sub>	1.807	"	1.35
L <sub>2</sub>	2189	"	1.642
L <sub>3</sub>	1530	"	1.15
L <sub>4</sub>	.553	"	.415
		$\Sigma =$	4.56 mg

To Page No. \_\_\_\_\_

99. The entries under "BSA" mg/ml" reflect the concentration of the serial dilutions of the protein standard I used to generate a standard curve for the protein. I measured the absorbance of the standards at 595 nm of BSA and recorded the absorbances for the standards under the "Abs 595" entry.

100. The entries labeled S<sub>1</sub> through S<sub>4</sub>, followed by “1:10”, correspond to the four elution fractions of GST-DD2-Short fusion protein diluted to a concentration of 1:10, and the entries labeled L<sub>1</sub> through L<sub>4</sub>, followed by “1:10”, correspond to the four elution fractions of GST-DD2-Long fusion protein diluted to a concentration of 1:10.

101. To the right of the fractions labeled S<sub>1</sub> through S<sub>4</sub> and L<sub>1</sub> through L<sub>4</sub>, I recorded the corresponding absorbance values for each of the samples.

102. At the far right side of page 88, I recorded the calculations I used to determine the protein concentration in each of the fractions corresponding to S<sub>1</sub> through S<sub>4</sub> and L<sub>1</sub> through L<sub>4</sub>. I calculated and listed the predicted concentrations of each sample under the entry “mg/ml” at the right side of the middle of page 88.

103. At the bottom of page 88, I calculated the total yield of protein (accounting for the 0.75 ml sample volume as reflected by the “x.75” entries), and recorded the total yield of proteins for the fractions under the “totals” entry at the bottom of page 88.

104. I noted at the bottom of page 88, that the sum of total protein for elution fractions S<sub>1</sub> through S<sub>4</sub> was 2.65 mg and the sum of total protein for samples L<sub>1</sub> through L<sub>4</sub> was 4.56 mg.

#### **9. Page 91, Notebook 26610**

105. I recorded and conducted the activities described on page 91 on April 15, 1997. I note that I wrote April 15, 1996 on page 91; however, the date should be April 15, 1997.

106. I recorded the title of the experiment on page 91 as “Silver Stain of GST-DD2 Protein” reflecting that I conducted a silver stain of small amounts of GST-DD2 fusion proteins that I ran on a gel. Silver stain binds to proteins and is a useful technique to confirm the presence of proteins such as the GST-DD2 proteins I eluted from the sepharose column as described on pages 89-90.



107. At the middle of page 91, I attached a photograph of the silver stain gel containing samples S<sub>1</sub> through S<sub>4</sub> (lanes 8-11) and L<sub>1</sub> through L<sub>4</sub> (lanes 3-6). Lanes 1 and 2 contained molecular weight markers and lanes 7 and 12 were empty as reflected in the description of the gel contents that I made at the bottom of page 91.

108. The results from the silver staining experiment confirmed that the expected size of the DD2-GST fusions were present in the elution fractions I prepared. The short form of the DD2-GST had a molecular weight of approximately 35 kilodaltons ("35kd") whereas the long form of the DD2-GST fusion had a molecular weight of approximately 40 kilodaltons ("40kd"). I recorded these results toward the bottom of page 91.

**10. Page 96, Notebook 26610**

109. I recorded and conducted the activities described on page 96 between April 16 and 18, 1997. There is no date on page 96, however the experiment I started on page 96 is further described on page 1, of my notebook 27510 which I dated April 18, 1997.

110. The experiment on page 96 is the start of a new series of experiments using plasmids that Scot Marsters provided to me that contained DNA encoding an Apo-2 receptor-Flag fusion insert. At approximately this time, the nomenclature used in the Ashkenazi lab to refer to the Apo-2 molecule began to shift from "DD2" to "Apo-2."

111. At the top of page 96, I made the following entries:

From Page No. _____					based on 6000 bp solution	
$A_{260}$		Plasmid				
1	.139	(21)	1.39 mg/ml		0.928 mg/ml	
2	.200	(38)	2.0 mg/ml		1.32 mg/ml	
3	.206	(56)	2.06 mg/ml		1.36	
4	.120	(81)	1.20 mg/ml		.792	

112. The entries above reflect that I quantified the amount of plasmid I received for each of the plasmids numbered "21", "38", "56", and "81" (corresponding to my numbering 1, 2, 3, and 4, respectively). I recorded the absorbance of the plasmid preparations at 260 nanometers ( $A_{260}$ ) and estimated the concentration for each plasmid. Scot Marsters provided the designations for the plasmids numbered "21", "38", "56", and "81."

113. At the middle of page 96, I made the entries in the image below reflecting that I ethanol precipitated the plasmid samples and re-solubilized the DNA precipitate in distilled water.

all of these were ethanol ppt & re-solubilized in dH<sub>2</sub>O to 1 ug / 7.5 ul or

	$\frac{133 \mu\text{g}}{\text{mL}}$			
	Total mass		Total	Brought up in ... dH <sub>2</sub> O
* 1	$.045 \text{ mL} \times .920 \frac{\text{mg}}{\text{mL}} =$		41.5 ug	312 uL
* 2	$.045 \text{ mL} \times 1.32 \frac{\text{mg}}{\text{mL}} =$		59.4 ug	447 uL
* 3	$.045 \text{ mL} \times 1.36 \frac{\text{mg}}{\text{mL}} =$		61.2 ug	460 uL
* 4	$.045 \text{ mL} \times .792 \frac{\text{mg}}{\text{mL}} =$		35.6 ug	268 uL

114. I conducted these activities as preliminary quality control activities using these plasmids prior to beginning any additional work using the plasmids. I noted at the bottom right of page 96 "1 of 27510" reflecting that page 1 of my notebook 27510 contained additional entries relating to the plasmids described on page 96.

**B. Relevant Entries From Notebook Number 27510 (ADE-21)**

**1. Page 1, Notebook 27510**

115. I recorded and conducted the activities described on page 1 on April 18, 1997. I note that I wrote April 18, 1996 on page 1; however, the date should be April 18, 1997.

116. The title of the experiment I described at the top of page 1 is "Apo2-ECD-Flag[g] In Vitro Translation of."

117. At the top of page 1, I wrote "Purpose: to look at in vitro translation of APO2-ECD Flag[g] Constructs 26610-96" reflecting that the purpose of the experiment was to evaluate the in vitro translation of Apo-2-ECD-Flag Constructs which I described on page 96 of my notebook 26610.

118. At the top of page 1, I wrote that plasmid numbers 21, 38, 56, and 81 “were all diluted to 133 ug/ml and used in the reticulocyte lysate TNT assay” indicating that the plasmids Scot Marsters gave me were subjected to an *in vitro* translation assay to express the proteins encoded in by the DNA in the plasmids numbered 21, 38, 56, and 81.

119. Following translation of the plasmids, I precipitated the lysate using a Flag-specific antibody. The Flag-specific antibody bound to the Flag portion of the fusion protein encoded by the plasmids Scot Marsters gave me and allowed for isolation of proteins expressing a Flag epitope.

120. I recorded the specific incubation and washing conditions at the middle of page 1 and further noted that following precipitation I ran the samples on a “4-20% gradient and SDS-PAGE.” I exposed the gel and noted at the bottom of page 1 that “All have translated #38 looks less, but is an artifact of lower load due to lab spill” reflecting that all of the plasmids Scot Marsters provided to me were successfully translated (although plasmid #38 appeared to produce less protein due to spillage of the sample before running the gel).

## **2. Page 2, Notebook 27510**

121. I recorded and conducted the activities described on page 2 on April 21, 1997.

122. I recorded the title of the experiment on page 2 as “Pulldown.”

123. I wrote the following at the top of page 2: “I. Purpose: to precipitate APO2L bound to *in vitro* translation expressed material.” My entry reflects that the purpose of this experiment was to precipitate Apo-2 ligand bound to the APO2-ECD-Flag *in vitro* translated products that I made on April 18 and described on page 1 of my notebook.

124. I made the following entries at the middle of page 2:

@ 10ug/ml			PPT
1. + Apo	DD2 ECD		Ni <sup>2+</sup>
2. -	DD2 ECD		"
3. +	DR4 - ECD		"
4. -	DR4 - ECD		"
5. +	CDC42 Flag		"
6. -	CDC42 Flag		"
7. +	CTRL UT		"
8. -	CTRL UT		"
9. -	CTRL UT		
912 -	DD2 ECD		αFlag.
1011 -	UT		αFlag.

125. The entries in the image above reflect that I set up an experiment where I added APO2 ligand ("APO") at a concentration of "10ug/ml" to various fusion proteins (e.g., DD2-ECD in sample 1).

126. The entries under "ppt" for samples 1-8 reflect that I used a nickel ("Ni<sup>2+</sup>") column to try to precipitate the various proteins, some of which I incubated with APO2-ligand. The Apo-2 ligand had a poly-histidine tag fused to it allowing use of a nickel column to bind APO2-ligand bound to the APO2/DD2 receptor.

127. I used a Flag specific antibody to try to precipitate proteins containing a Flag epitope for samples 9 and 10 as reflected by the "α-Flag" entry I made next to samples 9 and 10.

128. I did not record any results for this experiment in my notebook reflecting that the experiment was not successful.

### 3. Pages 4-5, Notebook 27510

129. I recorded and conducted the activities described on pages 4 and 5 on April 24, 1997, as reflected by the date I recorded on those pages next to my signature.

130. I recorded the title of the experiment at the top of page 4 as “Purification of Flag DD2.” Additionally, at the top of page 4, I wrote “Purpose: to purify flag-DD2 for binding experiments.” These entries reflect that the purpose of the experiment I conducted and described on page 4 was to purify DD2-Flag fusion proteins. Scot Marsters provided me with the DD2-Flag fusion proteins (from cell supernatants) that I purified in this experiment.

131. At the middle of page 4 under the “procedure” entry that I made, I recorded the seven step purification protocol I followed. Generally, the procedure involved preparing a chromatography column containing a resin to which I then added the supernatants containing the DD2-Flag fusion proteins. I washed the column and eluted the protein fractions with 0.1 molar glycine at pH 3.5 at one minute intervals. I then assayed the collected fractions by measuring the absorbance of the fractions at 280 nm and ran a gel followed by silver staining of the fractions.

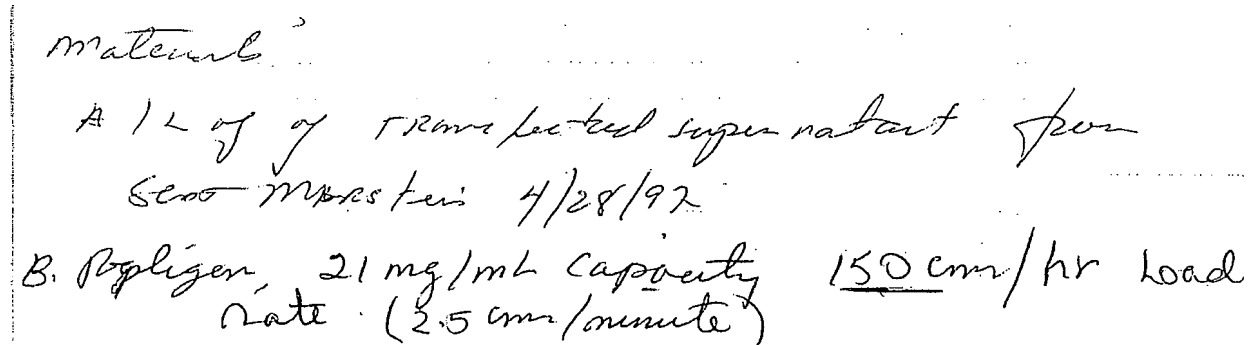
132. I taped the printout containing the chromatography results on page 5 of my notebook. At the middle of the printout, I wrote “Flag ECD DD2” and drew an arrow pointing to the first peak on the printout (reading from left to right). This entry reflects that this peak corresponds to the expected fraction containing the purified Flag-ECD-DD2 fusion protein indicating that the experiment was a success.

#### **4. Pages 8-10, Notebook 27510**

133. I recorded and conducted the activities described on pages 8-10 on April 29, 1997 as reflected by the date I recorded on those pages next to my signature.

134. At the top of page 8, I recorded the title of the experiment as “Apo2-IgG-Purification.” I wrote “Purpose: to prepare purified IgG-APO2” reflecting that the purpose of the experiment was to prepare purified fusion proteins made up of the APO2 receptor fused to an IgG fragment from an antibody.

135. At the top of page 8, I made the following entries under "Materials" as reflected in the image below. The entries reflect that I received one liter of transfected supernatant from Scot Marsters on April 28, 1997. The supernatant contained the Apo-2-IgG fusion proteins that I purified in this set of experiments. These entries also reflect that I used "Repligen", which is a resin used to purify proteins having IgG moieties, in the purification experiment.



The image shows a handwritten note in a notebook. The heading "materials" is written in cursive. Below it, two entries are listed: "A 1 L of transfected supernatant from Scot Marsters 4/28/97" and "B. Repligen, 21 mg/mL capacity 150 cm/hr load rate (2.5 cm/minute)".

materials

A 1 L of transfected supernatant from Scot Marsters 4/28/97

B. Repligen, 21 mg/mL capacity 150 cm/hr load rate (2.5 cm/minute)

136. At the middle of page 8, I wrote "Procedure" and listed the four steps that I followed when I conducted the purification of the APO2-IgG fusions. I ran the samples over the Repligen purification column according to the procedure I wrote on page 8.

137. I taped the chromatography printout I obtained from the purification described on page 8, to page 9 and labeled the printout "APO2-IgG" with a date of "4/29/97." The first large peak, reading from left to right, corresponds to the purified APO2-IgG fraction.

138. On page 10, I conducted a confirmatory total protein assay using the APO2-IgG fusion fraction I purified as described on pages 8 and 9.

139. I recorded the title of the experiment as "Purification APO2-IgG." The purpose of the assay was to determine the total protein yield in the fraction containing the purified APO2-IgG fusion protein.

140. To evaluate the protein content in the fraction, I conducted a Bradford assay, which is the generic name for the Biorad assay which I described earlier. Briefly, I added a

reagent to the fraction samples (labeled 6, 7, and 8 at the top of page 10) and measured the absorbances of the samples in a spectrophotometer at 595 nanometers. I compared the absorbances of the samples with the absorbances of a BSA protein standard curve. Based on the data available from the BSA standard curve, I was able to perform calculations and predict the protein content in the samples.

141. Based on the data I recorded on page 10, I predicted that the total yield of Apo-2 IgG fusion protein was 5.68 mg. I recorded my calculations and results at the bottom of page 10.

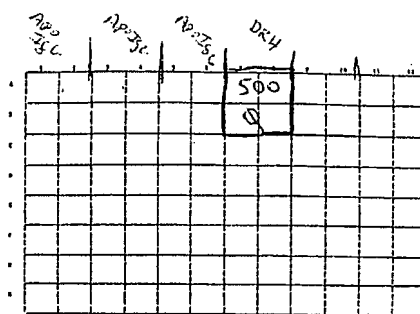
**5. Page 17, Notebook 27510**

142. I recorded and conducted the activities described on page 17 on April 29, 1997 as reflected by the date I recorded on that page next to my signature.

143. I recorded the title of this experiment as “<sup>125</sup>I-Apo-2-L Binding to Apo-2 IgG and Apo-2 Flag” reflecting that the experiment I described on page 17 is a binding experiment to evaluate the interaction of radiolabeled Apo-2-L with the Apo-2 receptor-IgG fusion protein and the APO2-FLAG fusion proteins.

144. The grid at the top portion of page 17 is a schematic of a 96 well microtiter plate which I used in these solid-phase type binding experiments. I wrote “APO-IgG” above columns 1-6 and “DR4” above columns 7 and 8, reflecting that I would include Apo-2-IgG and DR4 in those respective wells.





145. At the middle of page 17, I listed the concentration and dilutions of a FLAG-specific antibody (“ $\alpha$ -FLAG”), which was specific for the FLAG portion of the APO2-FLAG fusion protein, and a goat anti-Fc antibody, which was specific for the IgG portion of the APO2-IgG fusion protein.

146. At the bottom of page I listed the dilutions of both the non-radiolabeled Apo-2-L (“cold”) and the dilutions of the radiolabeled Apo-2-L (“hot”). Generally, the cold ligand is used to compete with hot ligand to determine whether the ligand specifically binds to the receptor.

147. My notebook entries ended abruptly and I did not record any results for this experiment reflecting that the experiment was unsuccessful.

#### 6. Page 21-26, Notebook 27510

148. The activities described on pages 21-26 were recorded and conducted on May 1, 1997 as reflected by the date I recorded on those pages next to my signature.

149. The experiments described on pages 21-26 all relate to conducting binding studies using a radiolabeled APO2-ligand and an APO2-IgG fusion protein. The fusion protein I used is the same as the one I purified from the supernatants Scot Marsters provided to me on April 28, 1997.

150. I recorded the title of the experiment on page 21 “Apo-2L Iodination.” I also wrote “Purpose to lactoperoxidase label APO2L” reflecting that my activities were directed to radiolabeling the APO2-ligand with radioactive iodine ( $^{125}\text{I}$ ) using the lactoperoxidase method for iodination.

151. At the top of page 21 next to entry “A.”, I made and recorded my calculations to determine the molar concentration of APO2L to be used in the iodination reaction. I wrote next to entry “B.”, “See following page for iodination procedure and calculation” reflecting that page 22 contained the lactoperoxidase protocol and the calculations I used to make the radiolabeled APO2L.

152. I taped the lactoperoxidase protocol sheet to page 22. I wrote APO2L next to the “Protein” entry on the sheet reflecting that I used the APO2L protein in the iodination reaction. I followed the procedure set forth under the “Procedure” section to make the labeled APO2L and recorded my calculations that I used to conduct the reaction at the bottom of page 22. I wrote “5/1/97” next to the “Date of Iodination” entry at the bottom of the protocol reflecting that I made the radiolabeled APO2L on May 1, 1997.

153. Once I made the APO2L label, I then conducted APO2-IgG-APO2L binding experiments as described on pages 23-26.

154. At the top of page 23, I recorded the title of this experiment as “Apo2-IgG/Apo-2-LI $^{125}$  Binding Assay.” I also wrote “I. Purpose: to bind APO2LI $^{125}$  to APO2-IgG in vitro” reflecting that the purpose of the experiment was to evaluate the binding of the radiolabeled APO2L to the APO2 receptor-IgG fusion protein.

155. At the middle of page 23, I made and recorded my calculations for both the “hot” and “cold” APO2L. I noted that the final concentration of hot ligand was 65 picomolar

("65pM"). The purpose of using cold (non-labeled) ligand is to compete with the hot ligand to evaluate the dissociation constant ( $K_d$ ) of the APO2 receptor with its ligand.

156. On page 24, I recorded additional activities directed to the binding assay I first described on page 23 of my notebook. I recorded the title of the experiment at the top of page 24: "Apo-IgG/Apo-2-LI<sup>125</sup> Binding Experiment" reflecting that this is a continuation of the binding experiments described on page 23.

157. I taped a printout of three microtiter plates to page 24 and labeled the plates as reflected in the image from page 24 below. The notations on the printout of the plates are my shorthand for the procedures that I followed in the binding experiment. A more precise explanation of the procedure I followed when I conducted the binding assay is set forth on the right side of page 25.

158. I listed the procedure I followed to conduct the binding assay under the "Procedure" entry on page 25.

159. At entry "1." under the "Procedure" I wrote that I coated the assay plates with a 1:200 dilution of "goat  $\alpha$ -human Fc", which is an Fc specific antibody. Coating the plates with an Fc specific antibody facilitates binding the IgG epitope of the APO2-IgG fusion protein. The Fc antibody binds to the plate and then 'captures' IgG proteins incubated in the wells. The Fc antibody-IgG protein is then available for binding with other reagents, such as APO2L.

160. At entry "2." under "Procedure" I wrote that I washed and blocked the plates. Washing removes any unbound Fc antibody and "blocking" the plates helps to reduce non-specific binding of other reagents added to the reaction.

161. At entry "3." under "Procedure" I wrote that I loaded the wells in the plates with "10ug/ml APO2-IgG" and incubated the plates for one hour at room temperature on a shaker. I expected the APO2-IgG to bind to the Fc antibody that I previously coated to the assay plates.

162. At entry "4." under "Procedure" I wrote that I washed the plates "3X" which removed unbound APO2-IgG, and then added the radiolabeled APO2L and non-radiolabeled ("cold") APO2L. I recorded the final concentration of the label as 65 picomolar on page 24 and used a serial dilution of cold APO2L at nanomolar concentrations to compete with the radiolabeled APO2L. (The concentrations are reflected in the entries at the middle plate on page 24).

163. At entry "5." under "Procedure" I wrote that the plates were washed "5X with wash buffer and then counted" reflecting that I washed the plates washed and measured the amount of radioactivity in each of the wells. I taped the data sheet containing the counts per minute ("cpm") to the left side of page 25 and taped two plots of the data I obtained to page 26.

164. The data I obtained reflect that APO2L binds with specificity to the APO2-receptor IgG fusion protein. Both plots I taped to page 26 reflect that the cold APO2L competed with the radiolabeled APO2L for binding to the APO2-IgG fusion protein. This is reflected by a decrease in counts per minute (a measure of the amount of radioactive APO2L bound to the APO2-IgG fusion) as the amount of non-labeled APO2L is added. The excess of cold APO2L competed with the APO2L label resulting in more occupation of binding sites by cold APO2L than by the APO2L label and therefore, a reduction in the cpms per sample.

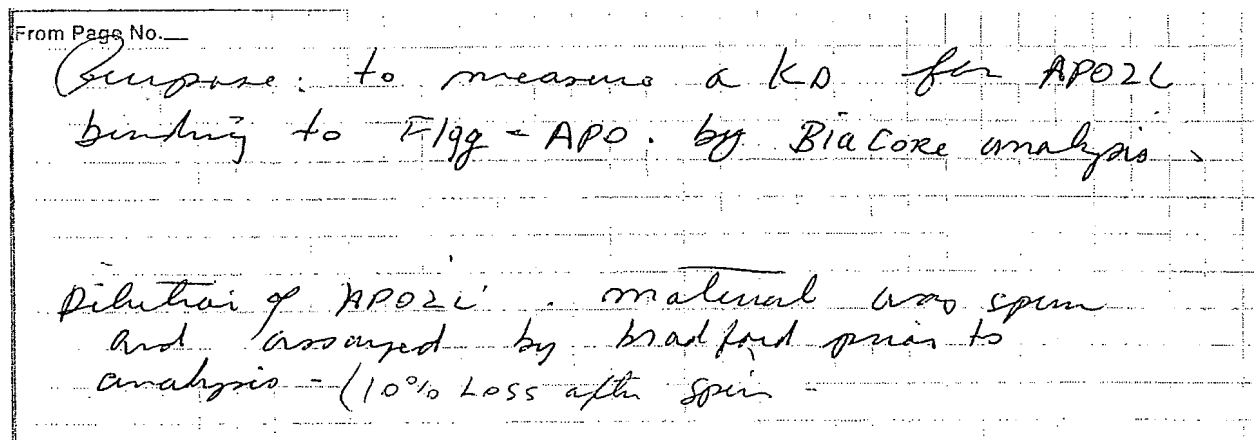
## **7. Page 27, Notebook 27510**

165. The activities described on page 27 were recorded and conducted on May 2, 1997.

166. The title of this experiment was "Biacore." Biacore was an approach to conduct receptor binding assays using biochips having the receptor crosslinked to the chip and then

flowing a ligand over the chip and evaluating receptor-ligand interactions. My intention in this experiment was to use the Biacore technology to evaluate the interactions of APO2L and APO2-ECD-Flag fusion proteins.

167. At the top of page 27, I made the following entries as reflected in the image below:



168. The purpose of this experiment was to measure a  $K_d$  (dissociation constant) for Apo-2-L binding to Flag-Apo2. The Biacore chips required significantly less protein to conduct the analyses which would help conserve resources.

169. I wrote that the Apo2-L material was centrifuged (“spun down”) and assayed for protein content using a Bradford Assay prior to conducting the binding assay using the Biacore chips.

170. I did not make any additional entries for this experiment.

8. Pages 28-29, Notebook 27510

171. I recorded and conducted the activities described on pages 28 and 29 on May 5, 1997 as reflected by the date I recorded on that page next to my signature.

172. I recorded the title of the experiment as: “Solid Phase Binding Apo-2-LI<sup>125</sup> to Apo2-IgG.” One of my goals in this set of experiments was to make a saturation curve for

APO2L binding to the APO2-IgG fusion protein. The saturation point of a receptor is when there are no longer available binding sites for the ligand and a saturation curve has an “S” shape. I taped a printout of the data I obtained and a saturation plot for the data I obtained at the bottom of page 29. The theory behind a saturation experiment is to use serial dilutions of the radiolabeled-ligand to evaluate the concentration of ligand to saturate the receptor as well as the amount required to reach the half saturation point or “half max.” The half-max for the APO2-IgG receptor binding APO2L-I<sup>125</sup> was 5.13 nM as reflected in the plot of the data I obtained at the bottom of page 29.

173. At the middle of page 28, I taped a printout of two assay plates, which is schematic of the reaction conditions I conducted for the saturation experiment. At the bottom of page 28 made several notes describing the saturation experiment I conducted. Under the entry “Saturation” I wrote the concentrations of the label I used to generate the saturation curve. I also noted that I “serially diluted” samples which would provide the data points for the middle part of the typical saturation curve, such as the one I taped to page 29.

174. My calculations are recorded in the entries at the bottom of page 29 as reflected in the image below.

Calculations  
A. Saturation  
400 µl of react labeled + 400 µl of 1:40 (of 840 ng/ml APO2L)  
serially diluted 1:1.5  
400 I<sup>125</sup> APO2L + 400 µl in 1:40 dilution → 500 + 250 etc

175. I made additional entries relating to the experiments on page 28, on page 29. I recorded the title of the experiment on page 29 as “Apo-2-LI<sup>125</sup> to Apo2IgG Solid Phase Binding.” At letter “B.” on page 29, I wrote “Calculations for competition” reflecting that I

conducted a binding competition experiment using the labeled APO2L and APO2-IgG fusion. I noted that I diluted the APO2L label (“hot”) at a 1:20 dilution and the cold at a 1:10 dilution followed by a serial dilution.

176. I conducted the competition assay and taped the raw data and a plot of the data I obtained to page 29 (“Competition: Apo-2-L-Apo125I/Apo2Ig”). The data reflect that the cold APO2L competed the APO2L label from the APO2-IgG fusion protein in a dose-dependent manner.

**9. Pages 32-33 Notebook 27510**

177. I recorded and conducted the activities described on pages 32 and 33 on May 6, 1997 as reflected by the date I recorded on those pages next to my signature.

178. The title of this experiment was “Binding DR4-IgG/Apo2IgG” reflecting that I conducted binding experiments using a DR4-IgG fusion as well as an APO2-IgG fusion. The purpose of the experiment was to evaluate the binding affinity of APO2L for the DR4-IgG and APO2-IgG and compare the binding characteristics of the receptors for the same ligand.

179. On page 32, I taped a printout of two assay plates that I used for the experiments. I used a plate corresponding to the description of the top plate to conduct binding studies to evaluate the binding specificity of the APO2-IgG and DR4-IgG fusions to four different ligands: LT $\alpha$  (lymphotoxin alpha), TNF  $\alpha$  (Tumor Necrosis Factor alpha), APO2L, and FASL (Fas ligand). I listed these ligands at the top of page 32. I used the plate described at the bottom to conduct competition studies using labeled and cold APO2L and the DR4-IgG or the APO2-IgG fusions.

180. The ligand specificity procedure involved incubating both the DR4-IgG and APO2-IgG fusions with radiolabeled APO2L and then using the other non-labeled ligands above

to compete with the APO2L label. If the non-labeled ligands were able to compete with the APO2L, it would suggest that the receptor had multiple specificity for different ligands.

181. I attached the data from the specificity experiment to the top of page 33 and the data reflect that the DR4-IgG and APO2-IgG are specific for the APO2L only and not for LT $\alpha$ , TNF $\alpha$ , or FASL.

182. I conducted the competition studies as I previously described. I incubated either the DR4-IgG or the APO2-IgG with labeled APO2L in the presence of serial dilutions of cold APO2L and evaluated whether an increase in the concentration of APO2L would compete with and displace the APO2L probe from the receptor.

183. At the middle of the page I taped the raw data and a plot of the data I obtained from the competition studies using the APO2-IgG and the DR4-IgG. The data reflect that the DR4-IgG and APO2-IgG have very similar binding characteristics and that cold APO2L specifically binds and competes to both the DR4 and APO2-IgG fusions.

**10. Page 34, Notebook 27510**

184. I recorded and conducted the activities described on page 34 on May 7, 1997 as reflected by the date I recorded on that page next to my signature.

185. I recorded the title of the experiment as "Repeat of Binding" and my notes include "APO-IgG" and "DR4-IgG" reflecting that the experiment I set up on page 34 was a repeat of the experiments I described on pages 32 and 33.

186. I wrote that I coated the assay plates with a 1:200 dilution of goat-anti-human Fc; that I added Apo2-IgG and DR4-IgG to the plates; and conducted a competition experiment.

187. I did not make any additional entries relating to this experiment.

**11. Page 36, Notebook 27510**

188. The activities described on page 36 were recorded and conducted on May 8, 1997.



189. The title of this experiment was "Biacore" reflecting that I was attempting to use the Biacore chip procedure to conduct binding assays.

190. At the middle of page 36, I made the following entries reflecting that I directly coupled the APO2-IgG fusion and the DR4-IgG fusion to individual Biacore chips. This involved linking the fusion proteins to the chip substrate which would then allow for subsequent incubation of the coupled receptors to ligands or other molecules of interest.

Direct Coupling

APO2-IgG

$\frac{2680}{50} = 1:50 \text{ dilution}$

DR4-IgG

$\frac{1480}{50} = 1:30 \text{ dilution}$

5/8 F.C 4

191. I wrote "5/8 F.C 4" on page 36 reflecting that I used flow cell number 4 to couple the APO2-IgG and DR4-IgG proteins to the chips.

192. The chips I prepared could be used for later binding experiments.

**12. Pages 38-39, Notebook 27510**

193. I recorded and conducted the activities described on page 38 on May 8, 1997 as reflected by the date I recorded on those pages next to my signature.

194. I recorded the title of the experiment as "Solid Phase Binding" reflecting that this is another binding experiment. The data from the experiment that I taped to page 39 reflect that I conducted another competition assay.

195. I noted at the top of page 38 “1:200 Goat  $\alpha$  Fc coat in carbonate” reflecting that I coated assay plates with a goat anti-Fc antibody. This is the same antibody that I used in all the solid phase binding experiments as the “capture” antibody to bind to the IgG portion of the APO2-IgG fusion or the DR4-IgG fusion.

196. I wrote “both receptors” at the top of page 38 reflecting that I used both the DR4-IgG and APO2-IgG fusions in this experiment. This is confirmed by the data sheets I taped to page 39 that I labeled with the DR4-IgG or APO2-IgG designations. I used three different concentrations for each receptor in the binding experiments to evaluate the effect of receptor concentration on ligand binding characteristics.

197. As I described above, page 39 contains the data I obtained from these experiments. At the top of the page is the raw data and at the bottom are two plots of the data I obtained. I labeled the plot at the bottom left of page 39 “Competition Curves for APO2L-I125 Binding to APO2-IgG.” The data in the plot reflect that APO2L competes with the APO2L label in a dose dependent manner and that receptor concentrations I used in the experiment (20ug/ml, 10ug/ml, and 5ug/ml) has little effect on the binding characteristics of APO2L to the APO2-IgG fusion.

198. I labeled the plot at the bottom right of page 39 “Competition Curves for APO2L-I125 Binding to DR4-IgG.” The data in the plot reflect that APO2L competes with the APO2L label in a dose dependent manner and that receptor concentrations I used in the experiment (20ug/ml, 10ug/ml, and 5ug/ml) has little effect on the binding characteristics of APO2L to the DR4-IgG fusion.

**13. Pages 40-42, Notebook 27510**

199. I recorded and conducted the activities described on pages 40 and 41 on May 9 11, and 12, 1997, respectively, as reflected by the dates I recorded on those pages next to my signature.

200. At the top of page 40, I recorded the title of this experiment as “Biacore Apo-2-L in an Apo2-IgG.” I also wrote “Purpose: to determine KD of APO2L to APO-IgG” reflecting that the purpose of the experiment was to use the Biacore procedure to determine the  $K_d$  (dissociation constant) of APO2L from the APO2-IgG fusion.

201. At the middle of page 40, I recorded the reaction conditions I followed for the Biacore procedure and the dilutions of the APO2L I used to flow over the Biacore chips.

202. After a recent review of my records, it appears that I did not advance this experiment any further.

203. I noted at the top of page 41 “This is essentially a repeat of the previous” and I listed the serial dilutions of APO2L at samples numbered 1 through 8 at the top of page 41. I wrote “FC4” reflecting that I used flow cell number 4 to conduct the experiment.

204. After a recent review of my records, it appears that I did not advance this experiment any further.


205. I noted at the top of page 42 “FC4 again APO2IgG  $\approx$ 900 RN” and I listed the serial dilutions of APO2L at samples numbered 1 through 8 at the top of page 42. This entry reflects that I repeated the experiments I described on pages 40 and 41.

206. After a recent review of my records, it appears that I did not advance this experiment any further.

V. **Conclusion**

207. I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of any patents issuing from the above-identified application.

Date: 4/20/2007

  
Robert Pitti

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

CAMELLA W. ADAMS *et al.*

Application No. 10/052,798

Filed: November 2, 2001

For: Apo-2 RECEPTOR

)  
) Docket No.: 22338-00904/P1101R2D1  
)  
) Examiner: Eileen B. O'Hara  
)  
) Group Art Unit: 1646  
)  
) Declaration in Support of Request for  
) Declaration of Interference  
) Under 37 C.F.R. § 41.202  
)  
) **Expedited Handling Requested**  
)  
)

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Alexandria, VA 22313-1450

**DECLARATION OF JAMES SHERIDAN, Ph.D.**

**ADE- 5**  
USSN 10/052,798

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## **DECLARATION OF JAMES SHERIDAN, Ph.D.**

I, James Sheridan, declare and state as follows:

### **I. Introduction and Background**

1. I am a citizen of the United States and presently reside in Mountain View, California.

2. I am currently employed by Protein Design Labs, Inc. as a Staff Scientist in the Translational Medicine Department.

3. I received my Bachelor's of Arts degree in Chemistry and Biology from Sonoma State University. I received my Doctor of Philosophy in Pharmacology from Stanford University School of Medicine. My doctoral studies defined critical cell cycle events preceding the onset of apoptosis when tumor cells are treated with commonly used chemotherapeutic agents.

4. I was a Post-Doctoral Scientist at Genentech, Inc., South San Francisco, CA ("Genentech") in the Department of Molecular Oncology from September 1995 through July 1998. The focus of my research was the isolation/characterization of novel receptors and ligands in the Tumor Necrosis Factor (TNF) family.

5. Dr. Avi Ashkenazi was my supervisor during the time period September 1995 through July 1998.

6. In this declaration, I refer to pages in my laboratory notebooks submitted as **ADE-22** and **ADE-23**.

7. The handwriting on the cited notebook pages is my handwriting, unless otherwise noted.

8. The descriptions I provide in this declaration include images from portions of my notebook pages. The notebook pages are reproduced in their entirety in the exhibits submitted

herewith. The images embedded in this declaration are accurate reproductions of the notebook pages and are intended to help guide the reader in considering my notebook entries.

9. I understand that Genentech intends to file this declaration at the United States Patent and Trademark Office to establish activities that occurred prior to March 17, 1997, and up to May 15, 1997. I understand that March 17, 1997, is a date by which another company filed a patent application relating to the Apo-2 receptor (also known as DR5) and that May 15, 1997, is the date on which Genentech filed a patent application relating to the Apo-2 receptor. Dates preceding March 17, 1997, on documents cited in this declaration have been redacted.

10. In this declaration, I provide the following:

- a. An overview of the personnel with whom I interacted at Genentech during my employment during the years 1995-1998;
- b. An overview of the types of experiments that I conducted while working for Dr. Ashkenazi; and
- c. A detailed description of my activities relating to the work I conducted using the Apo-2 receptor.

## **II. Overview of the Personnel With Whom I Interacted At Genentech**

11. In the course of my work during 1995-1998, I routinely interacted with several Genentech scientists including: Dr. Ashkenazi, Scot Marsters, Maya Skubatch, and Robert Pitti. I routinely discussed my work and experimental results with these scientists during our interactions in the close proximity of the Ashkenazi lab. These scientists, including Dr. Ashkenazi, would also discuss their results and objectives with me.

12. Prior to March 17, 1997, I understood that Dr. Ashkenazi was searching for receptor molecules in the TNF family. I understood that Dr. Ashkenazi was searching for novel



receptor sequences based on structural similarities to previously identified receptors in the TNF family (*e.g.*, Apo-3 and Fas), including sequences for 1) cysteine-rich regions in the extracellular domain, 2) a transmembrane domain; and 3) an intracellular region containing a death domain.

13. I recall that prior to March 17, 1997, Dr. Ashkenazi searched an Incyte sequence database and identified DNA sequences that he considered to be similar to the sequence of the Apo-3 receptor.

14. Prior to March 17, 1997, I clearly understood Dr. Ashkenazi's objectives regarding the newly identified receptor molecules. I worked in the Ashkenazi laboratory and Dr. Ashkenazi and I often discussed the progress of my research and his research objectives. At that time, I understood that upon identifying a novel receptor sequence, Dr. Ashkenazi planned to conduct cloning and expression experiments as well as to prepare a fusion protein (Apo-2-IgG (immunoglobulin G)) to use in binding studies. Additionally, I understood that Dr. Ashkenazi planned to initiate experiments to prepare antibodies against the novel receptor, including the preparation of agonist and antagonist antibodies that would specifically bind to the extracellular domain of the newly identified receptor.

15. Specifically, prior to March 17, 1997, I understood that Dr. Ashkenazi had contemplated a method of inducing apoptosis in a DR5-expressing cell which involved exposing the cell to an effective amount of an Apo-2 agonist monoclonal antibody which (a) binds to a soluble extracellular domain sequence (amino acids 54 to 182) of an Apo-2 polypeptide and (b) induces apoptosis in a DR5-expressing cell.

16. Once Dr. Ashkenazi identified sequences in the Incyte database, I recall that he asked Scot Marsters to design PCR primers and DNA probes based on the sequences Dr. Ashkenazi identified. Mr. Marsters designed the primers, designated them "DD" primers and a

“DD” probe, and asked a research associate collaborating with the Ashkenazi laboratory, Lakshmi Ramakrishnan, to screen various cDNA libraries and isolate clones having structural similarities to the sequences Dr. Ashkenazi identified.

17. At this time, I was informed by Dr. Ashkenazi or Scot Marsters that Ms. Ramakrishnan isolated a clone designated “DD2.” The “DD2” designation is a reflection of Scot Marsters’ probe designation (“DD”) and number “2” was the second positive clone (out of four) identified by Ms. Ramakrishnan.

18. Prior to March 17, 1997, based on the available information relating to the Apo-2 receptor, Dr. Ashkenazi had fully contemplated the idea that monoclonal antibodies could be prepared that would specifically bind to the extracellular domain of the Apo-2 receptor and that such antibodies could be agonists of the Apo-2 receptor. I knew this because the development of monoclonal antibodies against the Apo-2 receptor was openly discussed among the scientists in the Ashkenazi laboratory.

19. Prior to March 17, 1997, Dr. Ashkenazi also understood that a possible agonistic effect of such an antibody would be the induction of apoptosis in the cell, but that other agonistic functions were also possible.

### **III. Overview Of My Work Relating To The Apo-2 Receptor**

20. My work in the Ashkenazi laboratory relating to the Apo-2 receptor involved transfecting HeLa and 293 cells with a pRK5-based plasmid containing DNA sequences, including a sequence I designated 2-1 which was later determined to encode the Apo-2 receptor (Apo-2). My work also involved assaying transfected cells for apoptosis based on cellular morphology or using fluorescence-activated cell sorting (FACS). I confirmed through my experiments that clone 2-1 induced apoptosis in transfected HeLa and 293 cells.

21. The nomenclature of the 2-1 clone evolved over time. The 2-1 clone was often referred to by those working on the Apo-2 receptor project as “DD.2,” “DD2,” “DD2-1” and/or “DDP.2.” Ultimately, once Scot Marsters determined that the receptor encoded by the 2-1 DNA bound Apo-2 ligand (Apo-2L), the nomenclature shifted to “Apo-2” receptor.

22. In some experiments involving transfected HeLa cells, I included caspase inhibitors, such as “CRMA,” “DEVD,” or “ZVAD,” to evaluate whether caspases were involved in the apoptotic signal transmitted by the Apo-2 receptor. I found that the caspase inhibitors CRMA, DEVD and ZVAD blocked apoptosis induction by Apo-2 in transfected HeLa cells.

23. In other experiments, I included dominant-negative mutant forms of adaptor proteins, such as “FADD,” to evaluate whether these dominant-negative mutant adaptor proteins inhibited apoptosis signaling by the Apo-2 receptor when co-transfected into HeLa cells with the DNA encoding the Apo-2 receptor. FADD is an adaptor protein that mediates apoptosis activation by CD95, TNFR1 and Apo-3/DR3. I found that co-transfection of cells with FADD-DN and the 2-1 construct reduced apoptosis.

24. In addition to studying apoptosis induction by Apo-2, I conducted binding studies using Apo-2L and a purified Apo-2 receptor extracellular domain (ECD)-Flag molecule and analyzed whether the Apo-2 receptor-ECD-Flag fusion protein could block apoptosis induced by Apo-2L. I found that the Apo-2 receptor-ECD-Flag fusion protein was capable of blocking apoptosis induction by Apo-2L.

25. I also studied the effects of an NFκB inhibitor, ALLN (N-acetyl-Leu-Leu-norleucinal) and a transcription inhibitor, cyclohexamide, on the level of Apo-2L-induced apoptosis in HeLa cells and analyzed apoptosis in the cells by FACS. I found that both ALLN and cyclohexamide increased the level of Apo-2L-induced apoptosis compared to non-treated

cells. The data indicated that Apo-2L induces NF $\kappa$ B-dependent gene expression that had a protective function in HeLa cells. The data also indicated that the Apo-2 receptor may mediate NF $\kappa$ B activity.

26. I conducted DNA fragmentation analysis experiments to evaluate whether 293 cells transfected with clone 2-1 could induce characteristic apoptotic DNA fragmentation (a.k.a., “DNA laddering”) alone, or in the presence of caspase inhibitors and other adaptor proteins. DNA fragmentation into nucleosomal size repeats is a well known indicator of apoptosis and occurs when chromosomal DNA degrades during apoptosis.

27. In addition, I performed a radiation hybrid (RH) panel analysis to determine the chromosomal localization of the human DR4 gene, a TNF family receptor that also binds Apo-2L. RH mapping was performed using a commercially available kit and PCR using a human-mouse cell radiation hybrid panel and primers based on the coding region of the DR4 cDNA. I found that DR4 is linked to the marker D8S2127 (with an LOD of 13.00), which maps to human chromosome 8p21, the same chromosome to which the Apo-2 receptor maps.

#### **IV. Detailed Description of My Laboratory Notebook Entries**

28. I describe my activities in general categories of experiments. Within the description of each category, I essentially describe my experiments in chronological order.

##### **A. Transfected 293 Cells Assayed by Cellular Morphology for Apoptosis**

##### **Page 80, Notebook 26508 (ADE-22)**

29. I recorded and conducted the activities described on page 80 of Notebook 26508 prior to March 17, 1997. I signed and dated the bottom of page 80 prior to March 17, 1997.

30. My records reflect that Scot Marsters provided me with 293 cells transformed with a plasmid containing “DD” DNA and asked that I view the cells under a phase microscope

to evaluate whether the cells were apoptotic. This clone was referred to as "DD" because the sequence was thought to encode a putative death domain. Scot Marsters made this designation.

31. Across the top of page 80, I made the following entry:

USE TO  
80 USE TO  
USE TO  
USE TO

Project No. \_\_\_\_\_  
Book No. 26508 TITLE \_\_\_\_\_

From Page No. \_\_\_\_\_

PHOTOGRAPHED APOPTOSIS INDUCED BY OVEREXPRESSION OF  
DEATH DOMAIN CONTAINING PLASMID FROM MOLECULAR PROBING  
INCYTE DATABASE.

32. My entries reflect that I photographed transformed apoptotic 293 cells caused by overexpression of a plasmid having DNA corresponding to sequences from the Incyte database. I taped the photographs into my notebook page which is described below.

33. Across the middle of page 80, I made the following entry:

1. CONTROL @ LOW MTC. (COLOR GREEN)

2. TRANSFECTED (APOPTOTIC) @ HIGH MTC. (COLOR GREEN)

3. TRANSFECTED (APOPTOTIC) @ HIGH MTC. (BLACK AND WHITE)

1. DD - 293 -

4. CONTROL PRK5

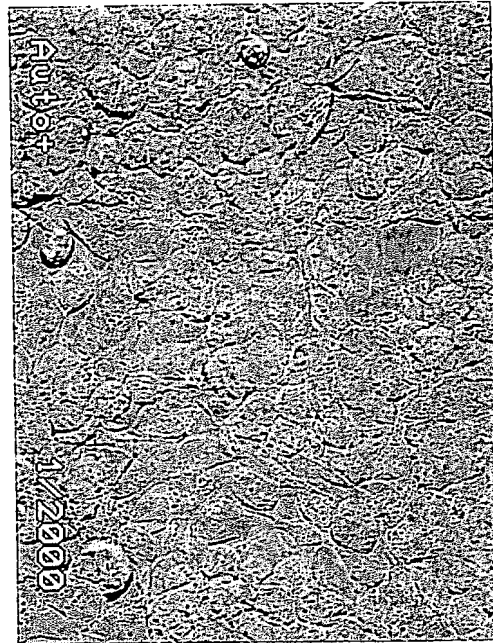
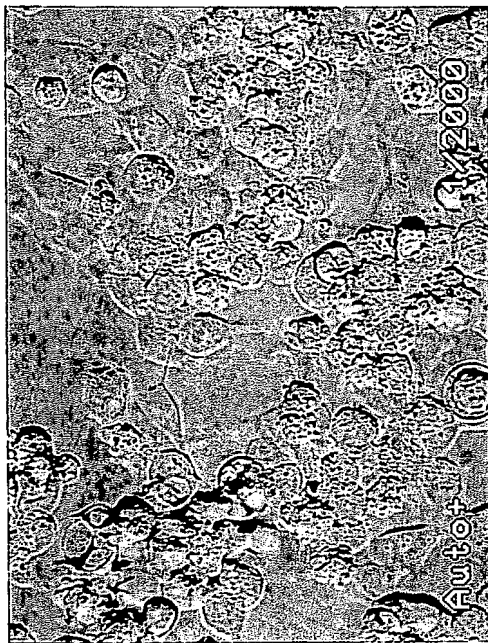
2. PRK5 - 293 - PRK5 -

34. My entries reflect that, when viewed under the phase microscope, the cells transfected with plasmid containing "DD" DNA exhibited cellular morphology indicative of apoptosis. For example, the cells easily came off the monolayer on the plate and exhibited classic membrane blebbing, as well as disintegrating nuclear envelopes.

35. I attached two black and white photographs to the middle of page 80, which are reproduced below. The photograph on the left depicts 293 cells that were transfected with the

plasmid containing the “DD” DNA. I photographed the cells at high magnification. The cells exhibited membrane blebbing, which is indicative of apoptosis.

36. The photograph on the right of page 80 corresponds to 293 cells that were transfected with a control pRK5 plasmid. These cells were not apoptotic.



37. I conveyed my observations to Scot Marsters and Dr. Ashkenazi soon after conducting these experiments and confirmed that the 293 cells transfected with the DD cDNA were apoptotic.

**Page 82, Notebook 26508 (ADE-22)**

38. I recorded and conducted the activities described on page 82 of Notebook 26508 prior to March 17, 1997.

39. My entries on page 82 reflect that Scot Marsters conducted transfection

experiments using 293 cells and vectors containing the DD DNA. The clone designation was “2-1.”

40. I taped a protocol sheet onto page 82 that described the various transfections of the 293 cells which Scot Marsters performed.

41. The roman numerals at the top of the protocol sheet refer to the allocation of the transfected 293 cells for four types of experiments which were performed within the Ashekanzi laboratory: 1) 500,000 cells for FACS analyses; 2) 500,000 cells for DNA Ladder experiments; 3) 1,000,000 cells for NFκB assay; and 4) 3,000,000 cells for SAP/c Jun assay.

42. Mr. Marsters transfected the 293 cells by calcium phosphate precipitation, as described in the “Title” section of page 82, and he gave the cells to me.

43. I assayed the transfected 293 cells for apoptosis based on cellular morphology 24 hours post-transfection with the 2-1 DNA.

44. I noted on the right side of the protocol sheet that apoptosis was “blocked” in sample number “5” corresponding to the cells co-transfected with the 2-1 construct and CRMA (a caspase inhibitor). My entry reflects that the 2-1 construct co-transfected with CRMA did not kill 293 cells following transfection, suggesting that the apoptotic signal mediated by the 2-1 DNA is caspase dependent.

45. The protocol sheet further reflects that Mr. Marsters co-transfected 293 cells with the 2-1 construct and FADD dominant negative protein (“FADD-DN (10 µg/ml)”). Mr. Marsters did this to evaluate whether the FADD adaptor protein mediated the apoptotic signal associated with the 2-1 protein. I noted that the co-transfected 293 cells with 2-1 construct and FADD-DN “maybe blocked” apoptosis.

46. The protocol sheet further reflects that Mr. Marsters co-transfected the 293 cells

with pRKRMA plasmid and pRK5 plasmid. I noted that the co-transfected 293 cells with the pRKRMA plasmid and pRK5 plasmid “blocked” apoptosis.

47. I did not record other results for this experiment on page 82.

**B. Transfected Hela Cells Assayed by FACS for Apoptosis**

**Page 83, Notebook 26508 (ADE-22)**

48. I recorded and conducted the activities described on page 83 of Notebook 26508 prior to March 17, 1997.

49. My records reflect that I conducted experiments in which I transfected HeLa cells with DNA from clones 1-1, 2-1, 3-1, and 4-1, which I understood to contain DNA sequences from the Incyte database. As previously noted, clone 2-1 was later designated the Apo-2 receptor.

50. The purpose of this experiment was to determine whether caspases were involved in the apoptotic signal associated with the DNA in clones 1-1, 2-1, 3-1 and 4-1.

51. Another purpose of the experiment was to evaluate whether adaptor proteins, such as FADD, TRADD and TRAF2, were involved in the apoptotic signal mediated by the protein encoded by the 2-1 construct.

52. I attached a copy of the protocol for this experiment to page 83 of my notebook, an image of which is provided below.



INCYTE constructs			R
Scot Marsters obtained possible clones of death domain containing constructs using the INCYTE Data Base on last Friday.			
Step 1 (Tuesday noon) Electroporate 1,000K HeLa cells with 8µg of gene X, 8µg of gene pRK5, 1µl VARNA, and 4µg of pRKCD4.			
Step 2 change medium 11 PM			
Step 3 Express for 24hrs and harvest for FACS. 4 PM WEDNESDAY			
✓ pRK5 (16µg/ml)		1	
✓ pRK5 (8µg/ml) 11 µl	1-1 (8µg/ml)	2	
✓ pRK5 (8µg/ml) 6 µl	2-1 (8µg/ml)	3	(killed 293s R)
✓ pRK5 (8µg/ml) 5 µl	3-1 (8µg/ml)	4	
✓ pRK5 (8µg/ml) 5 µl	4-1 (8µg/ml)	5	(may have killed 293s R)
(+) pRKCD4	6		
(+) pRK5	7		
(-) pRKCD4 (+) pRKCD4	8		
✓ pRK5 (8µg/ml) + CRMA (8µg/ml)		9	PE 25 Along
1-1 (8µg/ml) + CRMA		10	FITC FITC alone
2-1 (8µg/ml) + CRMA		11	PE + FITC
3-1 (8µg/ml) + CRMA		12	
4-1 (8µg/ml) + CRMA		13	
2-1 (8µg/ml) + MuFADD-DN (8µg/ml)		14	
2-1 (8µg/ml) + HuFADD-DN (8µg/ml)		15	
2-1 (8µg/ml) + Hu-TRADD-DN (8µg/ml)		16	
2-1 (8µg/ml) + Hu-TRAF2-DN (8µg/ml)		17	Did not do
Add DEVD 4hrs after electroporation			
pRK5 (16µg/ml)		+DEVD	18
pRK5 (8µg/ml) 2-1 (8µg/ml)		+DEVD	19
Add ALLN (100ng/ml) 4hrs before harvest			
pRK5 (16µg/ml)		+ALLN	20
pRK5 (8µg/ml) 2-1 (8µg/ml)		+ALLN	21

53. My entries reflect that I electroporated HeLa cells with 8 µg of gene X, 8 µg of gene Y, 1 µl VARNA and 4 µg of pRKCD4. The plasmid pRKCD4 was a marker I used to confirm that the transfection was successful (*i.e.*, that the cells took up plasmid DNA and now expressed the CD4 protein on their outer surface). I determined whether cells expressing plasmid encoded genes were apoptotic. I added the RNA stabilizer VARNA to increase the probability that all plasmid encoded genes would be expressed.

54. Each row of the protocol reflects the DNA constructs that I transfected into each

sample of HeLa cells. For example, I transfected sample #1 of the HeLa cells with 16 µg/ml of pRK5 (*i.e.*, vector only). Likewise, I transfected sample #2 of the HeLa cells with 8 µg/ml of pRK5 and 8 µg/ml of clone “1-1,” I transfected sample #3 of the HeLa cells with 8 µg/ml of pRK5 and 8 µg/ml of clone 2-1, etc. In this protocol, samples #6 through #8 were controls I used for calibrating the settings on the flow cytometer. Sample #6 contained cells transfected with plasmid pRKCD4. These transfectants were incubated with an antibody against CD4 that was conjugated to PE. PE is an abbreviation for phycoerythrin, a fluorescent dye used in FACS analyses. Sample #7 contained pRK5 transfected cells that were stained with annexin-FITC. Sample #8 contained pRKCD4 transfected cells with PE conjugate and FITC conjugate.

55. The viability of the cells was determined by fluorescence of FITC conjugated-annexin V binding to phosphatidylserine (PS). PS is expressed on the outside of cells when a cell is undergoing apoptosis. “FITC” is the abbreviation for fluorescein isothiocyanate and is a fluorescent dye used for FACS analysis. FITC is a small organic molecule, and is typically conjugated to the protein annexin-V. The Annexin-FITC conjugate binds to PS on the outside of the cell membrane allowing for detection of cells undergoing apoptosis.

56. Other vectors listed in the protocol included DNA encoding CRMA (a caspase inhibitor), MuFADD-DN (a murine dominant negative mutant of the FADD adaptor protein), HuFADD-DN (a human dominant negative mutant of the FADD adaptor protein), HuTRADD-DN (a human dominant negative mutant of the TRADD adaptor protein) and HuTRAF2-DN (a human dominant negative mutant of the TRAF2 adaptor protein).

57. I changed the medium of the plates 4 hours after electroporation. In future experiments, I added DEVD (a caspase inhibitor) 4 hours after electroporation to the plates or I added ALLN (an NFκB inhibitor) to the plates 4 hours before harvesting the cells. DEVD and

ALLN are small peptides that were added to the medium and diffused into the cells. I incubated the cells for 24 hours to express the transfected DNA constructs, harvested the cells from the plates and conducted FACS analyses. I did not conduct the transfection listed for samples #18 and #21.

58. I summarized my findings from the FACS analyses on the right side of page 83, an image of which is provided below. The percentage FITC positive staining cells that are also anti-CD4-PE positive cells represents the percentage of cells that were apoptotic and were also successfully transfected with plasmid DNA.

Project No. \_\_\_\_\_  
Book No. 26508

83

ANALYSIS RESULTS	% FITC POSITIVE
SAMPLE #	% PC POSIT.
#1	12.1% (i.e. TRANSFECTED AND APOPTOTIC)
#3	38.6% 35.4%
#9	12.9%
#11	11.4%
#14	48.5%
#15	28.9%
#16	42.5%
#17	50.8%
#18	
#19	54.9% 60.4%
#20	23.2%

59. My entries reflect some of the results from the FACS analyses. The left column of the entry shows the sample number. The far right column of the entry shows the percentage of cells that were FITC positive, *i.e.*, transfected cells that were apoptotic.

60. 14.1% of the cells in sample #1 (cells transfected with pRK5 vector alone) yielded a background level of apoptosis. 35.4% of the cells in sample #3 (cells transfected with pRK5 plasmid containing the 2-1 construct) were apoptotic. 12.9% of the cells in sample #9 (cells transfected with pRK5 vector and co-transfected with CRMA) were apoptotic, which was below the background percentage of apoptosis.

61. 11.4% of the cells in sample #11 (cells transfected with pRK5 plasmid containing the 2-1 construct and co-transfected with CRMA) were apoptotic, which was below the background percentage of apoptotic cells. 48.5% of the cells in sample #14 (cells transfected with pRK5 plasmid containing the 2-1 construct and co-transfected with MuFADD-DN) were apoptotic, which shows that MuFADD-DN did not block apoptosis. 28.9% of the cells in sample #15 (cells transfected with pRK5 plasmid containing the 2-1 construct and co-transfected with HuFADD-DN) were apoptotic, which shows that HuFADD-DN may have attenuated apoptosis.

62. 42.5% of the cells in sample #16 (cells transfected with pRK5 plasmid containing the 2-1 construct and co-transfected with Hu-TRADD-DN) were apoptotic, which shows that HuTRADD-DN did not block apoptosis. 50.8% of the cells in sample #17 (cells transfected with the 2-1 construct and co-transfected with Hu-TRAF2-DN) were apoptotic, which shows that Hu-TRAF2-DN did not block apoptosis. The results of samples 19 and 20 were impossible to interpret as corresponding control transfectants were not included in this experiment.

63. Based on my observations from the FACS analyses, I recorded my conclusions across the bottom of page 83, as reflected in the image below.

CONCLUSION:	OVEREXPRESSION OF 2-1 CAUSED APOPTOSIS
	- THIS COULD BE BLOCKED BY CRMA
	- THIS COULD BE REDUCED BY HU FADD-DN

64. My entries reflect that I concluded that, "Overexpression of 2-1 caused apoptosis. This could be blocked by CRMA. This could be reduced by HuFADD-DN." These entries reflect that cells transfected with clone 2-1 caused apoptosis, that the response could be blocked by the caspase inhibitor CRMA, and that the response could be reduced by co-transfection with the human dominant negative FADD mutant.

**Page 88, Notebook 26508 (ADE-22)**

65. I recorded and conducted the activities described on page 88 of Notebook 26508 prior to March 17, 1997.

66. My entry reflects that the title of the experiment described on page 88 was "Incyte construct 2-1."

67. I attached a typed protocol sheet to page 88 reflecting that I conducted transfection experiments using the 2-1 construct and HeLa cells. The protocol is detailed in the sheet attached to page 88. The experiment I conducted on this page was similar to the experiment I conducted and described on page 83 of Notebook 26508. I evaluated the effects of Apo-2 on apoptosis of HeLa cells transfected with vector alone (pRK5; samples 1 and 2); vector (pRK5) and the 2-1 construct (samples 3-12); and the 2-1 construct alone (samples 13 and 14). I also co-transfected cells with a pRKCD4 construct for use in identifying those cells that had successfully been transfected with plasmid DNA.

68. My entries reflect that I changed the medium of the plates 4 hours after electroporation. I incubated the cells for 24 hours to express the transfected DNA constructs, harvested the cells from the plates and conducted FACS analyses.

69. I did not record any results from this experiment on this notebook page.

**Page 90, Notebook 26508 (ADE-22)**

70. I recorded and conducted the activities described on page 90 of Notebook 26508 prior to March 17, 1997. The experiment described on page 90 is similar to the experiment I conducted and described on page 88 of Notebook 26508.

71. My entry reflects that the title of the experiment described on page 90 was "Incyte construct 2-1."

72. I attached a typed protocol sheet to page 90 reflecting that I conducted transfection experiments using the 2-1 construct and HeLa cells. The protocol is detailed in the sheet attached to page 90. Specifically, the experiments I conducted on this page were designed to evaluate the effects of Apo-2 on apoptosis of HeLa cells transfected with vector alone (pRK5; samples 1 and 2); vector (pRK5) and the 2-1 construct (samples 3-12); and the 2-1 construct alone (samples 13 and 14). I also transfected cells with a pRKCD4 construct for use in identifying those cells that had been successfully transfected with plasmid DNA.

73. My entries reflect that I added Apo-2L to the even numbered plates only and that I changed the medium of the plates 4 hours after electroporation. I incubated the cells for 24 hours to express the transfected DNA constructs, harvested the cells from the plates and conducted FACS analyses.

74. I recorded my results and noted the percentage of transfected HeLa cells that were apoptotic. Plates 7 and 8 (pRK5 (8 µg/ml) and 2-1 construct (8 µg/ml)) appeared to have the highest percentages of transfected and apoptotic cells (42.6% and 50.7%, respectively). Plate 8 contained Apo-2L, indicating that Apo-2L increased the percentage of apoptotic cells. The HeLa cells transfected with pRK5 vector alone (plate 1) yielded the lowest percentage of apoptosis

(16.6%).

75. I taped a graph into my notebook of the percentage of apoptotic HeLa cells transfected with the 2-1 construct in the presence and absence of Apo-2L. The data reflect that there was a higher percentage of transfected apoptotic cells in the presence of Apo-2L than without Apo-2L, suggesting that the receptor to which Apo-2L binds mediates apoptosis.

**Page 92, Notebook 26508 (ADE-22)**

76. I recorded and conducted the activities described on page 92 of Notebook 26508 prior to March 17, 1997. The experiment described on page 92 is similar to the experiment I conducted and described on page 83 of Notebook 26508.

77. My entry reflects that I recorded the title of the experiment described on page 92 as "Incyte construct 2-1."

78. I attached a typed protocol sheet to page 92 reflecting that I transfected HeLa cells with the 2-1 construct. Specifically, the experiments I conducted on this page were designed to evaluate the effects of Apo-2 on apoptosis of HeLa cells transfected with vector alone (pRK5; samples 1 and 2); vector (pRK5) and the 2-1 construct (samples 3 and 4); vector (pRK5) and CRMA (samples 5 and 6); the 2-1 construct and CRMA (samples 7 and 8); the 2-1 construct and murine FADD-DN mutant (samples 9 and 10); the 2-1 construct and human FADD-DN mutant (samples 11 and 12); the 2-1 construct and human TRADD-DN mutant (samples 13 and 14); the 2-1 construct and human TRAF2-DN mutant (samples 15 and 16); the 2-1 construct and human RIP-DN mutant (samples 17 through 19); vector (pRK5) alone incubated with DEVD (sample 20); vector (pRK5) and the 2-1 construct incubated with DEVD (sample 21); vector (pRK5) alone incubated with ALLN (sample 22); vector (pRK5) and the 2-1 construct incubated with ALLN (sample 23); vector (pRK5) alone incubated with cyclohexamide (sample 24); and vector

(pRK5) and the 2-1 construct incubated with cyclohexamide (sample 25). “RIP-DN” refers to the Dominant Negative form of RIP. RIP is an adaptor molecule that, in addition to a death domain, contains an N-terminal region that is homologous to Ser/Thr protein kinases. RIP possesses kinase activity as it autophosphorylates itself on Ser/Thr residues and overexpression of RIP engages the death pathway and activates NFκB.

79. My entries reflect that I changed the medium of the plates 4 hours after electroporation. I added DEVD 4 hours after electroporation to samples #20 and #21. I added ALLN 4 hours before harvest to samples #22 and #23. I added cyclohexamide 4 hours before harvest to samples #24 and #25. I incubated the cells for 24 hours to express the transfected DNA constructs, harvested the cells from the plates and conducted FACS analyses.

80. My handwritten notations reflect the final results from the experiments in terms of the percentage of transfected HeLa cells that were apoptotic.

81. 8.6% and 9.7% of the cells in samples #1 and #2, respectively (cells transfected with pRK5 vector alone) were apoptotic. 29.8% and 28.6% of the cells in samples #3 and #4, respectively (cells transfected with pRK5 plasmid containing the 2-1 construct) were apoptotic.

82. 19.3% and 16.6% of the cells in samples #5 and #6, respectively (cells transfected with pRK5 vector and co-transfected with CRMA) were apoptotic, which shows that CRMA was increasing the background apoptosis in the HeLa cells. 35.8% and 39.6% of the cells in samples #7 and #8, respectively (cells transfected with pRK5 plasmid containing the 2-1 construct and co-transfected with CRMA) were apoptotic, which shows that CRMA did not block apoptosis.

83. 37.5% and 40.7% of the cells in samples #9 and #10, respectively (cells transfected with pRK5 plasmid containing the 2-1 construct and co-transfected with MuFADD-DN) were apoptotic, which shows that MuFADD-DN did not block apoptosis. 14.9% and 15.7%



of the cells in samples #11 and #12, respectively (cells transfected with pRK5 plasmid containing the 2-1 construct and co-transfected with HuFADD-DN) were apoptotic, which shows that HuFADD-DN blocked apoptosis.

84. 41.0% and 40.4% of the cells in samples #13 and #14, respectively (cells transfected with pRK5 plasmid containing the 2-1 construct and co-transfected with Hu-TRADD-DN) were apoptotic, which shows that HuTRADD-DN did not block apoptosis. 44.1% and 43.7% of the cells in samples #15 and #16, respectively (cells transfected with the 2-1 construct and co-transfected with Hu-TRAF2-DN) were apoptotic, which shows that Hu-TRAF2-DN did not block apoptosis.

85. 49.7% and 52.7% of the cells in samples #17 and #18, respectively (cells transfected with the 2-1 construct and co-transfected with Hu-RIP-DN) were apoptotic, which shows that Hu-RIP-DN did not block apoptosis. I did not record any results for sample #19. 17.4% and 37.6% of the cells in samples #20 and #21, respectively (cells transfected with pRK5 (sample #20) or pRK5 with the 2-1 construct (sample #21) and both incubated with DEVD) were apoptotic, which shows that DEVD blocked apoptosis of sample #20 but not of sample #21.

86. 14.6% and 36.3% of the cells in samples #22 and #23, respectively (cells transfected with pRK5 vector alone (sample #22) or pRK5 with the 2-1 construct (sample #23) and both incubated with ALLN) were apoptotic, which shows that ALLN blocked apoptosis of sample #22 but not of sample #23.

87. 24.7% and 46.6% of the cells in samples #24 and #25, respectively (cells transfected with pRK5 vector alone (sample #24) or pRK5 with the 2-1 construct (sample #25) and both incubated with cyclohexamide) were apoptotic, which shows that cyclohexamide was associated with an increase in apoptosis when cells were successfully transfected with the 2-1

construct.

**Page 2, Notebook 27250 (ADE-23)**

88. I recorded and conducted the activities described on page 2 of Notebook 27250 on March 17 and 18, 1997. This experiment is similar to the experiment I described and conducted on page 92 of Notebook 26508.

89. I attached a typed protocol sheet to page 2 reflecting that I transfected HeLa cells using the 2-1 construct. The protocol is detailed in the sheet I attached to this page. Specifically, the experiments I conducted on this page were designed to evaluate the effects of Apo-2 on apoptosis of HeLa cells transfected with vector alone (pRK5; sample number 1); vector (pRK5) and the 2-1 construct (sample 2); the 2-1 construct co-transfected with CRMA (samples 3 and 4); the 2-1 construct and human FADD-DN mutant (samples 5 and 6); and the 2-1 construct and human RIP-DN mutant (sample 7).

90. I added YVAD 4 hours after electroporation to samples 8 and 9 and I added DEVD to sample 10. YVAD and DEVD are caspase inhibitors. Sample 8 contained cells transfected with vector (pRK5) alone and samples 9 and 10 contained cells transfected with pRK5 and the 2-1 construct.

91. I changed the medium of the plates 4 hours after electroporation. I incubated the cells for 24 hours to express the transfected DNA constructs, harvested the cells from the plates and conducted FACS analyses.

92. The numbers I wrote immediately to the right of the plate numbers reflect the percentage of apoptotic cells on each plate. The greatest percentage of apoptotic cells was in plate #2 (43.1%; pRK5 with the 2-1 construct) and plate #7 (41.9%; the 2-1 construct co-transfected with Hu-RIP-DN). The smallest percentage of apoptotic cells was in plate #9 (9.3%;

prK5 incubated with YVAD).

**Page 4, Notebook 27250 (ADE-23)**

93. I believe I recorded and conducted the activities described on page 4 of Notebook 27250 between March 19 and 21, 1997. Although page 4 is not dated, I recorded the previous date of the experiment on page 3 as March 19, 1997. I dated the subsequent experiment on page 5 as March 21, 1997. This experiment is similar to the experiment I conducted and described on page 2 of Notebook 27250.

94. I attached a typed protocol sheet to page 4 reflecting that I conducted transfection experiments using the 2-1 construct and HeLa cells. The protocol is detailed in the sheet attached to page 4. Specifically, the experiments I conducted on this page were designed to evaluate the effects of Apo-2 on apoptosis of HeLa cells transfected with vector alone (pRK5; sample # 1); vector (pRK5) and the 2-1 construct (sample #2); the 2-1 construct co-transfected with CRMA (samples #3 and #4); and the 2-1 construct con-transfected with human FADD-DN mutant (samples #5 and #6).

95. My entries reflect that I added DEVD 4 hours after electroporation to samples #7 and #8 and I added ZVAD to samples #9 and #10. Samples #7 through #10 contained cells transfected with prK5 and the 2-1 construct. DEVD and ZVAD are caspase inhibitors.

96. My entries reflect that I changed the medium of the plates 4 hours after electroporation. I incubated the cells for 24 hours to express the transfected DNA constructs, harvested the cells from the plates and conducted FACS analyses.

97. My entries reflect that I noted that, "Background apoptosis was too high. I used unhealthy HeLa cells in the transfection."

**Page 8, Notebook 27250 (ADE-23)**

98. I believe I recorded and conducted the activities described on page 8 of Notebook 27250 on March 21 and March 22, 1997. Although page 8 is not dated, I recorded the previous date of the experiment on page 7 and the subsequent experiment on page 9 as March 21, 1997. This experiment is similar to the experiment I conducted and described on page 2 of Notebook 27250.

99. I attached a typed protocol sheet to page 8 reflecting that I conducted transfection experiments using the 2-1 construct and HeLa cells. Specifically, the experiments I conducted on this page were designed to evaluate the effects of overexpressing the Apo-2 receptor on apoptosis of HeLa cells transfected with vector alone (pRK5; sample #1); vector (pRK5) and the 2-1 construct (sample #2); the 2-1 construct and CRMA (samples #3 and #4); the 2-1 construct and human FADD-DN mutant (samples #5 and #6); and the 2-1 construct and human-RIP-DN (sample #7).

100. My entries reflect that I added DEVD 4 hours after electroporation to samples #8 and #9 and I added YVAD to samples #10 through #12. Sample #8 through #12 contained cells transfected with pRK5 and the 2-1 construct.

101. My entries reflect that I changed the medium 4 hours after electroporation of the plates. I incubated the cells for 24 hours to express the transfected DNA constructs, harvested the cells from the plates and conducted FACS analyses.

102. My handwritten notation reflects the percentage of cells that were apoptotic. Cells in sample #2 (29.2%; pRK5 and the 2-1 construct) and sample #7 (38.5%; the 2-1 construct co-transfected with Hu-RIP-DN) had the highest percentage of apoptosis. Apoptosis was not blocked by Hu-RIP-DN.

**Page 12, Notebook 27250 (ADE-23)**

103. I believe I recorded and conducted the activities described on the top of page 12 of Notebook 27250 between March 21 through March 24, 1997. Although page 12 is not dated, I recorded the previous date of the experiment on page 10 as March 21, 1997. I dated the subsequent experiment on page 13 as March 24, 1997. This experiment is similar to the experiment I conducted and described on page 2 of Notebook 27250.

104. I attached a typed protocol sheet to page 12 reflecting that I transfected HeLa cells using the 2-1 construct. Specifically, the experiments I conducted on this page were designed to evaluate the effects of Apo-2 on apoptosis of HeLa cells transfected with vector alone (pRK5; sample #1); vector (pRK5) and the 2-1 construct (samples #2 and #3); the 2-1 construct and human FADD-DN mutant (samples #4, #5 and #6); and the 2-1 construct and human-RIP-DN (samples #7 and #8). I also transfected cells with a pRKCD4 construct for use as a positive control.

105. My entries reflect that I changed the medium 4 hours after electroporation. I incubated the cells for 24 hours to express the transfected DNA constructs, harvested the cells from the plates and conducted FACS analyses.

106. My handwritten notation to the right of the sample number reflects the percentage of cells that were apoptotic. Samples #7 and #8 (the 2-1 construct and Hu-RIP-DN) had the highest percentage of apoptosis of 42.9% and 40.9%, respectively.

107. On the bottom of page 12, I describe the results continued from page 1 dated March 17, 1997. This was an experiment to measure DNA fragmentation caused by apoptosis.

**Page 20, Notebook 27250 (ADE-23)**

108. I believe I recorded and conducted the activities described on page 20 of

Notebook 27250 between April 2 and 3, 1997. Although page 20 is not dated, I saved the results of the analysis as “JS2040397,” corresponding to my first and last initials and 04/03/1997. This experiment is similar to the experiments I conducted and described on pages 2 and 12 of Notebook 27250.

109. I attached a typed protocol sheet to page 20 reflecting that I transfected HeLa cells using the 2-1 construct. Specifically, the experiments I conducted on this page were designed to evaluate the effects of Apo-2 receptor overexpression on apoptosis of HeLa cells transfected with the 2-1 construct and human FADD-DN mutant (plates 1 through 4) and pRK5 vector and human-FADD-DN (plates 5 through 8). All of the plates were co-transfected with pRK5 vector.

110. My entries reflect that I changed the medium of the plates 4 hours after electroporation. I incubated the cells for 24 hours to express the transfected DNA constructs, harvested the cells from the plates and conducted FACS analyses.

111. My handwritten notation reflects the percentage of cells that were apoptotic. Cells in samples #1 and #2 had lower percentages of apoptosis of 19.7% and 21.0%, respectively, due to the higher concentration of Hu-FADD-DN (inhibitor of apoptosis) than the cells in samples #3 and #4. The cells in samples #3 and #4 had percentages of apoptosis of 30.6% and 29.8%, respectively.

**Page 21, Notebook 27250 (ADE-23)**

112. I believe I recorded and conducted the activities described on page 21 of Notebook 27250 between April 2 and 3, 1997. Although page 21 is not dated, I saved the results of the analysis as “JS2040397,” corresponding to my first and last initials and 04/03/1997.

113. Page 21 of Notebook 27250 reflects the data I obtained from the transfection of HeLa cells described on page 20 of Notebook 27250.

114. I attached two scatter plots and two histograms of sample A (CD4 PE) to page 21. The scatter plots and histograms show that sample 1 resulted in approximately 19.7% annexin-FITC positive cells among the CD4 positive population.

**Page 22, Notebook 27250 (ADE-23)**

115. I believe I recorded and conducted the activities described on page 22 of Notebook 27250 between April 4 and 5, 1997. Although page 22 is not dated, I saved the results of the analysis as "JS2040597," corresponding to my first and last initials and 04/05/1997. This experiment is similar to the experiments I conducted and described on pages 2, 12 and 20 of Notebook 27250.

116. I attached a typed protocol sheet to page 22 reflecting that I transfected HeLa cells using the 2-1 construct. Specifically, the experiments I conducted on page 22 were designed to evaluate the effects of Apo-2 on apoptosis of HeLa cells transfected with pRK5 vector alone (sample 1); the 2-1 construct and human FADD-DN mutant (samples 2 through 6); and the 2-1 construct and pRK5 vector (samples 7 through 11).

117. My entries reflect that I changed the medium 4 hours after electroporation. I incubated the cells for 24 hours to express the transfected DNA constructs, harvested the cells from the plates and conducted FACS analyses.

118. My handwritten notation to the right of the plate numbers reflects the percentage of cells that were apoptotic. Cells in samples #2 through #6 had lower percentages of apoptosis than cells in samples #7 through #11 because HuFADD-DN is an inhibitor of apoptosis.

119. Across the bottom of page 22, I attached a sheet containing scatter plots and histograms which I created using the data I obtained from the transfection of HeLa cells described above.

120. The scatter plots and histograms show that sample 1 resulted in approximately 11.5% annexin-FITC positive cells among the CD4 positive population.

**Page 25, Notebook 27250 (ADE-23)**

121. I recorded and conducted the activities described on page 25 on April 6, 1997. I signed and dated the bottom of page 25 with the date April 6, 1997.

122. I attached a summary sheet to page 25 of the data from the transfection of HeLa cells which I performed on pages 2, 4, 8, 12, 20 and 22 of notebook number 27250. The summary sheet is reflected by the image below.

pRK5	pRK5+2-1	HuFADD-DN+2-1	<del>Z-VAD</del> XVAD+2-1
9.4	29.8	22.4	9.3
11.6	28.6	20.3	11.5
8.6	43.9	14.9	9.1
9.7	35.4	15.7	10.0 ± 1.3
14.1	43.1	28.9	
13	29.2	30.4	DEVD+2-1
11.5	31.9	32.1	
11.1 % ± 2.0	33.4	22.0	14.4
	36.5	25.7	17.2
	38.4	26.7	19.0
	41.7	20.6	16.9 ± 2.3
	36.0	20.4	
	36.3	24.2	
	35.7 ± 5.1	24.7	
		25.3	
		23.6 ± 4.9	
MuFADD-DN+2-1	HuTRADD-DN+2-1	HuTRAF2-DN+2-1	
37.1	41.0	50.8	
40.7	40.0	44.1	
48.5	42.5	43.7	
42.2 ± 5.6	41.2 ± 1.2	46.2 ± 4.0	
pRKRMA+2-1	HuRIP-DN+2-1		
11.4	49.7		
16.7	52.7		
11.3	41.9		
13.1 ± 3.1	38.5		
	42.9		
	40.9		
	44.4 ± 5.5		

123. The data from these scatter plots and histograms shows the percentage apoptosis from the transfections of HeLa cells transfected with pRK5 alone; pRK5 co-transfected with the 2-1 construct; ZVAD co-transfected with the 2-1 construct; Murine FADD-DN co-transfected with the 2-1 construct; Human TRADD-DN co-transfected with the 2-1 construct; Human



TRAF2-DN co-transfected with the 2-1 construct; pRKRMA co-transfected with the 2-1 construct; and Human RIP-DN co-transfected with the 2-1 construct.

124. The highest average percentage of apoptotic cells occurred in HeLa cells co-transfected with the 2-1 construct and Human TRAF2-DN (46.2 +/- 4.0%); the 2-1 construct and Human RIP-DN (44.4 +/- 5.5%); and the 2-1 construct and Murine FADD-DN (42.2 +/- 5.6%). HeLa cells co-transfected with the 2-1 construct and ZVAD yielded the lowest average percentage of apoptotic cells of 10.0 +/- 1.3%.

### C. Binding Interaction Between Apo-2 and Apo-2L

#### Page 41, Notebook 27250 (ADE-23)

125. I conducted the activities described on page 41 of Notebook 27250 on April 22 and 23, 1997. I recorded the activities on April 24, 1997. I signed and dated the bottom of page 41 with the date April 24, 1997.

126. My entries reflect that on April 22, 1997, I plated HeLa cells (10 mM EDTA) with 100,000 cells per well in 12 well dishes.

127. I attached a protocol sheet to page 41 detailing an apoptosis competition assay using HeLa cells, as reflected by the image below.

TITLE _____	Book No. <u>27250</u>	41
04-23-97		
Plated HeLa cells (10mMEDTA) @ 100K cells/well in 12 well dishes previous evening. 10:30 HAM pre-incubate the Apo2L (make a 1µg/ml stock using 3.3 µl in 3 ml) +/- ECD (100nM) and +/- FLAG MAb (1µg/µl-dilute to 1µg/µl by adding 5µl to 10µl medium) for 1hr. 11:30 → 4:30	MAKF 3.2 OF 1.1/1.1 µl APO2L STOCK 6.6 µl 3.2	

128. My entries reflect that on April 23, 1997, I pre-incubated Apo-2L (stock of 1 µg/ml) in the presence and absence of the extracellular domain of Apo-2 (Apo-2 ECD) (100 nM) and in the presence and absence of Flag monoclonal antibody (mAb) (diluted to 1 µg/ml by

adding 5 µl to 10 µl medium) for 1 hour. I harvested the cells and performed FACS analyses to measure apoptosis.

129. I attached the image below to page 41, which is the remainder of the protocol sheet detailing the apoptosis competition assay using HeLa cells.

Pre-incubate the cells in cycloheximide 1 hr at 50µg/ml.

I *no cycloheximide*

1 <i>64.9%</i>	0.5 µg/ml	Apo2L +	Tris Buffer (50µl)	+ Anti-Flag Mab (1µg/ml)
2 <i>44.9%</i>	0.3µg/ml	Apo2L +	Tris Buffer (50µl)	+ Anti-Flag Mab (1µg/ml)
3 <i>12.5</i>	NT	+	Tris Buffer (50µl)	+ Anti-Flag Mab (1µg/ml)
4 <i>13.4%</i>	0.5 µg/ml	Apo2L +	ECD (50µl)	+ Anti-Flag Mab (1µg/ml)
5 <i>11.0</i>	0.3µg/ml	Apo2L +	ECD (50µl)	+ Anti-Flag Mab (1µg/ml)
6 <i>11.4</i>	NT	+	ECD (50µl)	+ Anti-Flag Mab (1µg/ml)

II *~ 4 µg/ml OF ECD PROTEIN*

Preincubate and include cycloheximide

7 <i>31.4%</i>	0.03 µg/ml	Apo2L +	Tris Buffer (50µl)	+ Anti-Flag Mab (1µg/ml)
8 <i>15.5</i>	0.01 µg/ml	Apo2L +	Tris Buffer (50µl)	+ Anti-Flag Mab (1µg/ml)
9 <i>15.0</i>	NT	+	Tris Buffer (50µl)	+ Anti-Flag Mab (1µg/ml)
10 <i>13.7%</i>	0.03 µg/ml	Apo2L +	ECD (50µl)	+ Anti-Flag Mab (1µg/ml)
11 <i>13.9</i>	0.01 µg/ml	Apo2L +	ECD (50µl)	+ Anti-Flag Mab (1µg/ml)
12 <i>14.7</i>	NT	+	ECD (50µl)	+ Anti-Flag Mab (1µg/ml)

Kodak Anti-Flag M2 Mab 3µg/µl stock.

130. The left column lists plates 1 through 12. The numbers immediately to the right of the plate numbers are the percentage of apoptotic cells. The remainder of the columns show the concentrations of Apo-2L, Tris buffer or ECD and anti-Flag MAb that I added to each plate. "NT" means that Apo-2L was not added.

131. I did not pre-incubate the first group of plates (plates 1 through 6) with cyclohexamide. I did pre-incubate the second group of plates (plates 7 through 12) with cyclohexamide (50 µg/ml) for 1 hour.

132. I described the anti-Flag monoclonal antibody as "Kodak Anti-Flag M2 Mab 3  $\mu\text{g/ml}$  stock."

133. Cells that I incubated with and without cyclohexamide in the presence of Apo-2L, ECD and anti-Flag mAB were the least apoptotic (plates 4-5 and 10-11). The Apo-2-ECD-Flag appeared to block apoptosis induced by Apo-2L. The cells incubated without cyclohexamide in the presence of Apo-2L, Tris buffer and anti-Flag mAB were the most apoptotic (plates 1-2). The Apo-2-ECD-Flag was not present to block apoptosis induced by Apo-2L.

**Page 48, Notebook 27250 (ADE-23)**

134. I conducted the activities described on page 48 of Notebook 27250 on April 28 and 29, 1997. I recorded the activities described on page 48 of Notebook 27250 on April 29, 1997.

135. My entries reflect that on April 28, 1997, I plated HeLa cells at 250,000 cells per well in 12 well dishes.

136. I attached a protocol sheet to page 48 detailing the activities I performed, as reflected in the image below.

04-29-97 FC IC50	
Plated HeLa cells (10mMEDTA) @ 250Kcells/well in 12 well dishes previous evening. Preincubate the Apo2L (0.55 $\mu\text{l}$ /0.5ml) +/-Apo2 Fc for 1hr.	
1 A	Apo2L (1 $\mu\text{g/ml}$ ) + Fc (27 $\mu\text{g/ml}$ )
2 B	Apo2L (1 $\mu\text{g/ml}$ ) + Fc (9 $\mu\text{g/ml}$ )
3 C	Apo2L (1 $\mu\text{g/ml}$ ) + Fc (3 $\mu\text{g/ml}$ )
4 D	Apo2L (1 $\mu\text{g/ml}$ ) + Fc (1 $\mu\text{g/ml}$ )
5 E	Apo2L (1 $\mu\text{g/ml}$ ) + Fc (.33 $\mu\text{g/ml}$ )
6 F	Apo2L (1 $\mu\text{g/ml}$ ) + Fc (.11 $\mu\text{g/ml}$ )
7 G	Apo2L (1 $\mu\text{g/ml}$ ) + Fc (.037 $\mu\text{g/ml}$ )
8 H	Apo2L (1 $\mu\text{g/ml}$ ) + Fc (.012 $\mu\text{g/ml}$ )
9 I	Apo2L (1 $\mu\text{g/ml}$ ) + Fc (.004 $\mu\text{g/ml}$ )
10 J	Apo2L (1 $\mu\text{g/ml}$ ) + Fc (.0014 $\mu\text{g/ml}$ )
11 M	Apo2L (1 $\mu\text{g/ml}$ ) + Fc (.0004 $\mu\text{g/ml}$ )
12 N	Apo2L (1 $\mu\text{g/ml}$ ) + medium

*Handwritten notes:*  
10  $\mu\text{l}$  STRAIGHT  
3.3  $\mu\text{l}$   
1:30  $\Rightarrow$  6:30  
Split + 10  $\mu\text{l}$  MEDIUM  $\Rightarrow$  USE 10  $\mu\text{l}$  ADD 10  $\mu\text{l}$  cell...

137. My entries reflect that on April 29, 1997, I pre-incubated the cells with Apo-2L

(0.55  $\mu$ l/0.5ml) in the presence or absence of Apo2-Fc for 1 hour. Apo2-Fc is a fusion protein containing the Fc region of human immunoglobulin IgG heavy chain fused to the ECD of the Apo-2 receptor. I harvested the cells and performed FACS analyses.

138. In my entries above, the first column is the plate number and letter, plate 1A through 12N. In the second column, the concentration of Apo-2L and Apo2-Fc is shown in parentheses. Plate 12N did not contain Apo2-Fc.

139. Across the bottom of page 48, I noted the percentage of apoptotic cells from the above experiment, as reflected by the image below.

A	10.8%
B	10.1%
C	9.3%
D	11.4%
E	12.8%
G	15.0%
H	31.9%
J	47.4%
K	62.0%
L	60.6%
M	56.1%
N	64.1%

140. The left column is the letter designation for each plate and the right column is the percentage Apo-2L induced apoptosis in the cells. The highest percentage of apoptotic HeLa cells was found in plate N (64.1%) in which I incubated the HeLa cells with Apo-2L and medium, but without Apo2-Fc. The next highest percentage of apoptotic HeLa cells was found in plates K and L (62.0% and 60.6%, respectively.) Plate K contained HeLa cells incubated with Apo-2L and Apo2-Fc (0.004  $\mu$ g/ml), and Plate L contained HeLa cells incubated with Apo-2L

and Apo2-Fc (0.0014 µg/ml).

**Page 49, Notebook 27250 (ADE-23)**

141. I conducted the activities described on page 49 of Notebook 27250 on April 28 and 29, 1997. I recorded the activities described on page 49 of Notebook 27250 on April 29, 1997.

142. My entries reflect that on April 28, 1997, I plated HeLa cells (10 mM EDTA) at 150K, 200K, 250K and 300K cells per well in 12-well dishes.

143. I attached a protocol sheet to page 49 detailing the activities I performed, as reflected in the image below.

04-29-97

04-29-97

Plated HeLa cells (10mMEDTA) @ 150K, 200K, 250K, and 300K cells/well in 12 well dishes previous evening. The size of Apo2 is ~44Kd and the Fc should be about 100Kd, therefore use a 1:4 mass to mass ratio.

144. I attached the remainder of the protocol sheet to page 49 detailing the activities I performed, as reflected in the image below.

9AM pre-incubate the Apo2L (make a 1 µg/ml stock using 3.3 µl in 3 ml) +/- IgG/Fc (2 µg/ml) for 1 hr. Make a 0.25 µg/µl stock of Apo2 Fc by diluting in medium and use 5 µl/ well.

<b>A</b>	0	NT		8.2%	13 x 0.5 = 6.5
	1	Apo2L (0.5 µg/ml)		57.5%	
	2	Apo2L (0.5 µg/ml)		54.2%	7.2 µl = 6.5
	3	Apo2L (0.5 µg/ml)		57.4%	
<b>B</b>	I.	(20 µg/ml) Apo2L Fc (stock is 2.7 mg/ml)			
	4	Apo2L (0.5 µg/ml) + Apo2 Fc (3.7 µl)		8.5%	
	5	Apo2L (0.5 µg/ml) + Apo2 Fc (3.7 µl)		10.3%	
	6	Apo2L (0.5 µg/ml) + Apo2 Fc (3.7 µl)		10.6%	
	7	NT	+	Apo2 Fc (3.7 µl)	9.4%
	8	NT	+	Apo2 Fc (3.7 µl)	10.4%
	9	NT	+	Apo2 Fc (3.7 µl)	8.3%
	II.	20 µg/ml TNF IgG (dilute 1:10 Scot's 20 µg/µl stock in medium)			
	10	Apo2L (0.5 µg/ml) + TNFR1 IgG (5 µl)		62.6%	
	11	Apo2L (0.5 µg/ml) + TNFR1 IgG (5 µl)		62.9%	
	12	Apo2L (0.5 µg/ml) + TNFR1 IgG (5 µl)		65.3%	
	13	NT	+	TNFR1 IgG (5 µl)	10.8%
	14	NT	+	TNFR1 IgG (5 µl)	10.0%
	15	NT	+	TNFR1 IgG (5 µl)	10.1%
	III.	DR4 Fc			
	16	Apo2L (0.5 µg/ml) + DR4 Fc (50 µl)		25	17.1%
	17	Apo2L (0.5 µg/ml) + DR4 Fc (50 µl)		25	17.2%
	18	Apo2L (0.5 µg/ml) + DR4 Fc (50 µl)		25	16.9%
	19	NT	+	DR4 Fc (50 µl)	25 8.8%
	20	NT	+	DR4 Fc (50 µl)	25 10.5%
	21	NT	+	DR4 Fc (50 µl)	25 8.9%

145. My entries reflect that on April 29, 1997, I pre-incubated the HeLa cells with Apo-2L (stock of 1 µg/ml) in the presence and absence of IgG/Fc (2 µg/ml) for 1 hour. I made a 0.25 µg/ml stock of Apo-2-Fc by diluting in medium. I used 5 µl of Apo-2-Fc per well.

146. My entries reflect that after incubating the HeLa cells with Apo-2L in the presence or absence of IgG/Fc, I harvested the cells and measured apoptosis of the cells by performing FACS analyses.

147. For the purpose of this declaration, only the samples involving Apo-2L and Apo-2-Fc are discussed.

148. The notation at arrow "A" reflects that I did not add any Apo-2L-Fc (signified by "NT") to plate 0. I only added Apo-2L to plates 1 through 3. The column to the right of Apo-2L is the percentage of HeLa cells that were apoptotic.

149. The notation at arrow "B" reflects that I added Apo-2L and Apo-2-Fc to plates 4 through 6. I did not add Apo-2L (signified by "NT"), but added Apo-2-Fc to plates 7 through 9. The column to the right of Apo-2-Fc is the percentage of HeLa cells that were apoptotic.

150. The results showed that Apo-2-Fc inhibited the induction of apoptosis by Apo-2L (plates 4 through 6). These results showed that Apo-2-Fc specifically bound to Apo-2L.

151. Across the bottom of page 49, I noted that, "The Apo-2 Fc blocked Apo-2L induced apoptosis." The image below reflects this notation.

A photograph of a handwritten note on lined paper. The text is written in dark ink and reads: "M6 Apo2Fc BLOCKED APO2L INDUCED APOPTOSIS." The note is written on a piece of paper with horizontal lines, and the handwriting is in all caps.

**Page 56, Notebook 27250 (ADE-23)**

152. I conducted the activities described on page 56 of Notebook 27250 on April 28 and 29, 1997. I recorded the activities described on page 56 of Notebook 27250 on April 29, 1997.

153. My entries reflect that on April 28, 1997, I plated HeLa cells (10 mM EDTA) at 250K cells per well in 12-well dishes. This experiment is similar to the experiment I conducted and described on page 48 of Notebook 27250.

154. My entries reflect that on April 29, 1997, I pre-incubated the cells with Apo-2L (0.55  $\mu$ l/0.5ml) in the presence or absence of Apo2-Fc for 1 hour. I harvested the cells and

performed FACS analyses.

155. I noted the percent of apoptotic cells on page 56 from the experiment described above. The image below reflects this notation.

1.	6.9%
2.	8.1%
3.	6.7%
4.	6.7%
5.	10.2%
6.	15.6%
7.	20.4%
8.	26.3%
9.	33.8%
10.	41.0%
11.	42.8%
12.	49.0%

156. The left column is the plate designation and the right column is the percent Apo-2L induced apoptosis. The highest percentage of apoptotic HeLa cells was found in plate 12 (49.0%) in which I incubated the HeLa cells with Apo-2L and medium, but without Apo2-Fc. The next highest percentage of apoptotic HeLa cells was found in plates 11 and 10 (42.8% and 41.0%, respectively.) Plate 11 contained Hela cells incubated with Apo-2L and Apo-2-Fc (0.0004  $\mu\text{g/ml}$ ), and Plate 19 contained Hela cells incubated with Apo-2L and Apo-2-Fc (0.0014  $\mu\text{g/ml}$ ).

**D. The Effects of ALLN and Cyclohexamide on Apoptosis Induced by Apo-2L in HeLa Cells**

**Page 84, Notebook 26508 (ADE-22)**

157. I recorded and conducted the activities described on page 84 of Notebook 26508 prior to March 17, 1997.

158. I taped a protocol sheet to page 84 detailing the activities I conducted, which is



reflected in the image below.

84

Project No. 26508  
Book No.            TITLE           

From Page No.           

cyclohexamide:HeLa

HeLa: Apo2L, TNF, and LT (plated in HGD MEM)

Dose response in the presence and absence of cyclohexamide. Plate ~200K cells in 6 well plates on the previous night. Cells will be pretreated with ALLN for 1 hr prior to Apo2L. Cells will be incubated with Apo2L for 5hrs prior to annexin staining.

8:00AM pretreat 12 wells +/- cyc. @ 40µg/ml (1:1000) for 1 hr.

10:00AM treat cells with each ligand @ 1µg/ml.

2:00PM Harvest and annexin stain (no PI).

~~293~~ H07  
Apo2L

37	cyc +	NT
38	cyc +	1:500
39	cyc +	1:1000
40	cyc +	1:2000
41	cyc +	1:4000
42	cyc +	1:8000
43		NT
44		1:500
45		1:1000
46		1:2000
47		1:4000
48		1:8000

159. My entries reflect that I plated 200,000 HeLa cells in 6 well plates (numbered 37 through 48) the previous night. The next day, I then treated half of the plates of HeLa cells (plates 37 through 42) with the transcription inhibitor cyclohexamide for one hour. The pretreatment of the HeLa cells with cyclohexamide was to shut down the production of survival

signaling proteins. I then incubated plates 38 through 42 and plates 44 through 48 with Apo-2L to evaluate the effects of cyclohexamide on apoptosis induced by Apo-2L.

160. My entries reflect that after a 5 hour incubation with Apo-2L, I harvested the cells and conducted the annexin FACS assay to determine whether the cells were apoptotic.

161. I calculated the percentage of apoptotic cells based on the population of HeLa cells that bound the annexin-FITC conjugate.

162. I made the following entries on page 84 based on calculations from the FACS analyses I conducted on the HeLa cells.

Apo-2L	
37	5.4%
38	8.2%
39	67.6%
40	59.1%
41	54.9%
42	42.7%
Cyc	
43	5.0%
44	35.8%
45	34.6%
46	25.3%
47	16.3%
48	5.1%

163. Samples 38 through 42 show the percentage of Apo-2L-induced apoptosis in HeLa cells incubated with cyclohexamide. When the survival signaling was eliminated by adding cyclohexamide, the cells became extremely sensitive to induction of apoptosis by Apo-2L.

164. Samples 44 through 48 show the percent of Apo-2L-induced apoptosis in the HeLa cells incubated without cyclohexamide. These samples show there is a dose response relationship by adding large amounts of Apo-2L to the surface of healthy HeLa cells. The data show that as the dilution of Apo-2L increases, apoptosis decreases. A comparison of the data from plates 38 through 42 reflect that incubation with cyclohexamide generally increased the level of Apo-2L-induced apoptosis in the HeLa cells.

**Page 85, Notebook 26508 (ADE-22)**

165. I recorded and conducted the activities described on page 85 of Notebook 26508 prior to March 17, 1997. This experiment is similar to the experiment I conducted and described on page 84 of Notebook 26508, except that in this experiment I added ALLN (an NFκB inhibitor) to the HeLa cells instead of cyclohexamide.

166. I calculated the percentage of apoptotic cells based on the total population of HeLa cells that bound the annexin-FITC conjugate.

167. I made the following entries on page 85 based on calculations from the FACS analyses I conducted on the HeLa cells.

1	4.8%
2	80.3%
3	64.0%
4	51.2%
5	34.4%
6	17.4%
7	6.6%
8	51.0%
9	31.6%
10	26.9%
11	18.4%
12	7.2%

168. The entries above reflect the data from the FACS analyses I conducted on the HeLa cells. The left column reflects the sample numbers. The right column reflects the percentage of cells that underwent apoptosis in response to the treatment with Apo-2L with or without ALLN pretreatment.

169. Samples 2 through 6 show the percentage of Apo-2L-induced apoptosis in HeLa cells incubated with ALLN. When NF $\kappa$ B activity was inhibited by adding ALLN, the cells became very sensitive to induction of apoptosis by Apo-2L.

170. Samples 8 through 12 show the percentage of Apo-2L-induced apoptosis in the HeLa cells incubated without ALLN. These data reflect the dose response of Apo-2L to the surface of healthy HeLa cells. The data reflect that incubation of the HeLa cells with ALLN increased the level of Apo-2-L-induced apoptosis in the HeLa cells.

**Page 7, Notebook 27250 (ADE-23)**

171. I conducted the activities described on page 7 of Notebook 27250 on March 20 and 21, 1997. I recorded the activities described on page 7 of Notebook 27250 on March 21, 1997. This experiment was similar to the experiment I conducted and described on page 84 of Notebook 26508.

172. My entries reflect that on March 20, 1997, I plated 200K HeLa cells in 6 well plates.

173. I taped the following entry on page 7 and handwrote the percentage of apoptotic cells.

<u>A</u>	<u>B</u>	<u>C</u>	<u>D</u>
		Apo2L	
1	0.4% cyc.		+ NT
2	0.5% cyc.		+1:500
3	2.9% cyc.		+1:1000
4	4.3% cyc.		+1:2000
5	4.6% cyc.		+1:4000
6	8.6% cyc.		+1:8000
7	7.3%		NT
8	72.3%		1:500
9	63.5%		1:1000
10	41.1%		1:2000
11	24.5%		1:4000
12	10.0%		1:8000

174. The column under arrow "A" reflects plate numbers 1 through 12 containing HeLa cells.

175. The column under arrow "B" reflects the data from the FACS analyses I conducted on the HeLa cells and indicates the percentage of cells that underwent apoptosis in response to the treatment with Apo-2L.

176. The column under arrow "C" indicates that plates 1 through 6 were pre-incubated with cyclohexamide. I performed this activity on March 21, 1997. Plates 7 through 12 were not pre-incubated with cyclohexamide as reflected by the absence of a "cyc" entry corresponding to these plates.

177. The column under arrow "D" shows the dilutions of Apo-2L that I added to the cells in plates 2-6 and 8-12. I performed this activity on March 21, 1997. I did not add any Apo-2L to plates 1 or 7 (designated as "NT").

178. My entries reflect that after a 5-hour incubation with Apo-2L, I harvested the cells

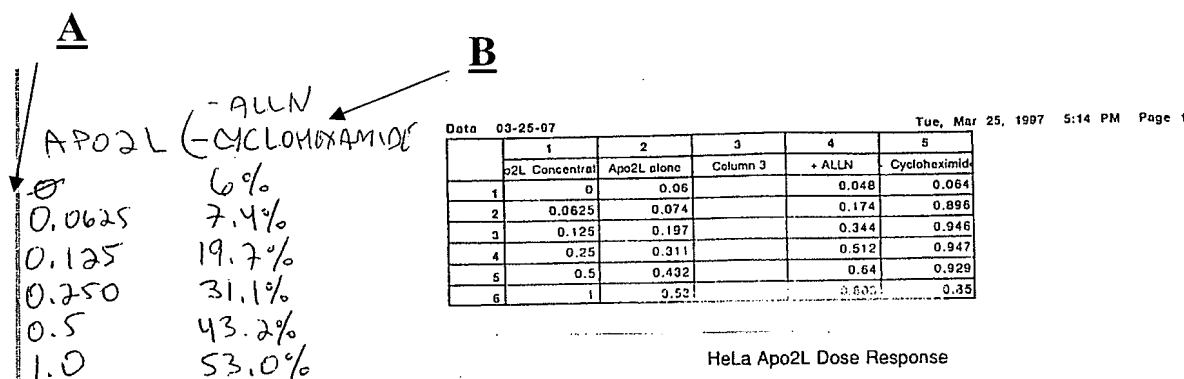
and I conducted the annexin FACS assay to determine whether the cells were apoptotic. The data reflect that cyclohexamide increased the level of Apo-2-L-induced apoptosis in HeLa cells.

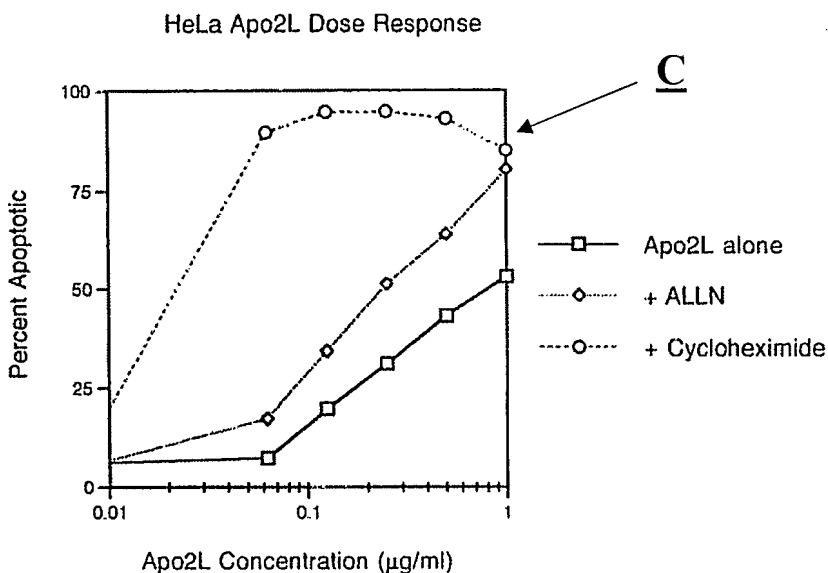
**Page 14, Notebook 27250 (ADE-23)**

179. I recorded and conducted the activities described on page 14 of Notebook 27250 on March 25, 1997.

180. The title of this page was "Summary of HeLa Dose Response 03-25-97 Using Data From JS 4 Pg. 85 & JS 5 Pg. 7." On page 14, I summarized the dose response data from the experiments I performed on page 85 of Notebook 26508 and again on page 7 of this notebook. On page 85 of Notebook 26508, I studied the dose response of HeLa cells in the presence of ALLN and Apo-2L. On page 7 of Notebook 27250, I studied the dose response of HeLa cells in the presence of cyclohexamide and Apo-2L.

181. I taped the following entry of a dose response chart and dose response curve to the top of page 14 and I added in my handwritten notes.





182. This entry shows the data collected from the dose response experiments performed on page 85 of Notebook 26508 (ALLN and Apo-2L) and page 7 of Notebook 27250 (cyclohexamide and Apo-2L).

183. Arrow “A” points to the Apo2L concentration. Arrow “B” points to the percentage of apoptotic cells without ALLN or cyclohexamide. Arrow “C” points to a graph showing a dose response curve of HeLa cells in the presence of Apo-2L alone, in the presence of Apo-2L and ALLN, and in the presence of Apo-2L and cyclohexamide. The graph shows that HeLa cells in the presence of Apo-2L and cyclohexamide resulted in the greatest percentage of apoptotic cells.

**Page 43, Notebook 27250 (ADE-23)**

184. I believe I recorded and conducted the activities described on page 43 of Notebook 27250 between April 24 and 25, 1997. Although page 43 is not dated, I saved the results of the analysis as “JS2042597,” corresponding to my first and last initials and 04/25/1997.

185. I taped a protocol sheet detailing the activities I conducted on page 43. My entries reflect that I plated 200K HeLa cells in 6 well plates on the previous night, before treating

the cells with ALLN or cyclohexamide.

186. My entries reflect that the next day, I treated HeLa cells with ALLN or cyclohexamide for one hour and then incubated the cells with Apo-2L for 5 hours to evaluate the effects of ALLN or cyclohexamide on apoptosis induced by Apo-2L. The protocol I followed is reflected in the image below.

(03-24-97)

HeLa (plated in HGDMEM)

Dose response in the presence and absence of ALLN (inhibitor of I $\kappa$ B degradation) or cycloheximide. Plate ~200K cells in 6 well plates on the previous night. Cells pretreated with ALLN or cycloheximide for 1 hr prior to Apo2L. Cells will be incubated with Apo2L for 5hrs prior to annexin staining.

8:00AM pretreat 12 wells +/- ALLN @ 40 $\mu$ g/ml (1:1000) for 1hr.

9:00AM treat cells with each ligand @ 1 $\mu$ g/ml.

0.5  $\mu$ g/ml

2:00PM Harvest and annexin stain (no PI).

1	NT	5.6%	
2	NT	5.1%	
3	NT	5.7%	8.0%
4	ALLN + NT		7.0%
5	ALLN + NT		7.4%
6	ALLN + NT	25.8%	
7	cyc. + NT	7.0%	
8	cyc. + NT	6.3%	
9	cyc. + NT	8.9%	
10	Apo2L 1 $\mu$ g/ml	0.5	25.5%
11	Apo2L 1 $\mu$ g/ml		29.0%
12	Apo2L 1 $\mu$ g/ml		30.6%
13	cyc. + Apo2L 1 $\mu$ g/ml	0.5 $\mu$ g/ml 51.9%	
14	cyc. + Apo2L 1 $\mu$ g/ml	58.9%	
15	cyc. + Apo2L 1 $\mu$ g/ml	61.8%	
16	ALLN + Apo2L 1 $\mu$ g/ml	0.5	58.2%
17	ALLN + Apo2L 1 $\mu$ g/ml		59.2%
18	ALLN + Apo2L 1 $\mu$ g/ml		61.3%

187. The first column reflects plate numbers 1 through 18 containing HeLa cells. The second column reflects what I added to each plate.

188. I did not pre-incubate plates 1 through 3 with ALLN or cyclohexamide and I did

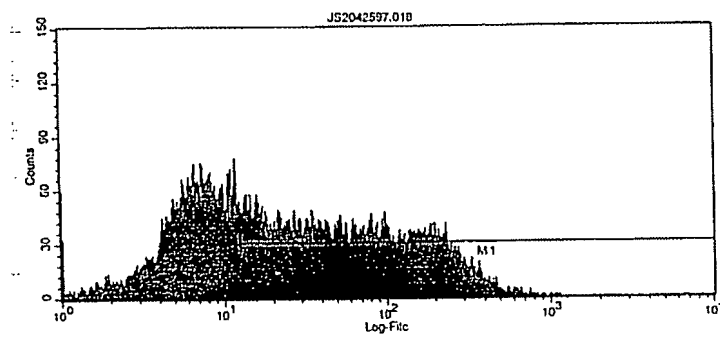
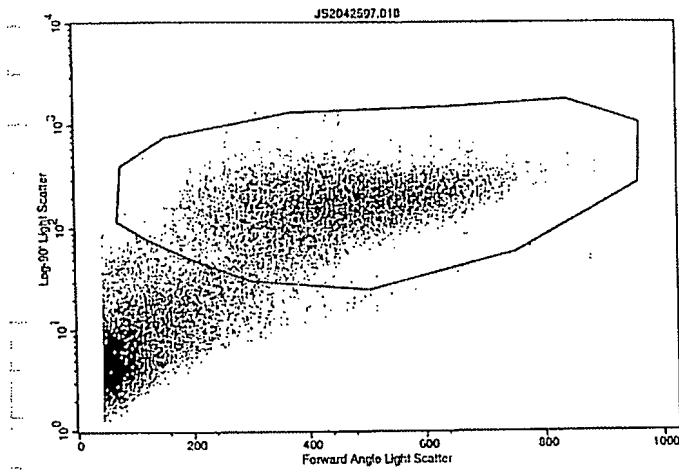


not treat these plates with Apo-2L, as reflected by the designation “NT”. I pre-incubated plates 4 through 6 with ALLN without Apo-2L (NT). I pre-incubated plates 7 through 9 with cyclohexamide without Apo-2L (NT). I incubated plates 10 through 12 with Apo-2L alone. I pre-incubated plates 13 through 15 with cyclohexamide and Apo-2L. I pre-incubated plates 16 through 18 with ALLN and Apo-2L. The parentheses next to Apo-2L show the concentration (0.5 µg/ml) at which I added Apo-2L.

189. My entries reflect that after a 5 hour incubation with Apo-2L, I harvested the cells and conducted the annexin FACS assay to determine whether the cells were apoptotic.

190. The percentages noted at the end of each row reflect the data from the FACS analyses I conducted on the HeLa cells and indicates the percentage of cells that underwent apoptosis in response to the treatment with cyclohexamide or ALLN and Apo-2L.

191. I taped scatter plots and histograms of the data from the dose response experiment across the right and bottom of page 43, as reflected by the images below. I also handwrote my notes from this experiment.



Sample ID: 18 Gated Events: 20203  
 Total Events: 45840

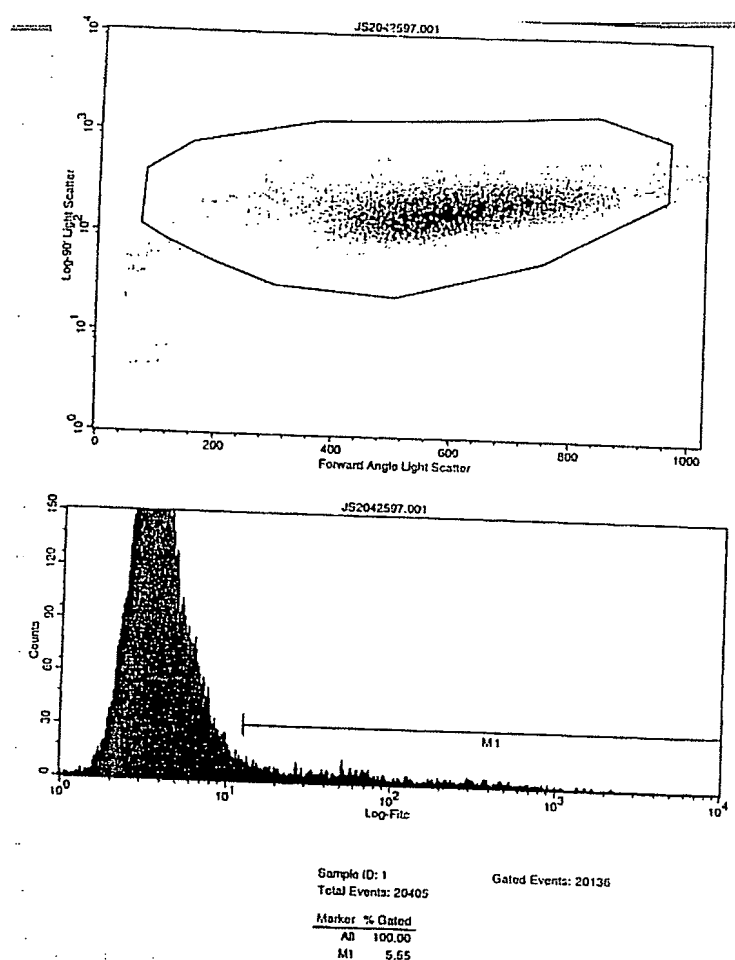
Marker	% Gated
All	100.00
M1	01.29

All 100.00  
 M1 5.55

THOSE PLOTS WERE  
 REALLY NOISY AND EACH  
 INDIVIDUAL CELL MAY HAVE  
 BEEN LESS DRUG THAN  
 USUAL (MEANING LESS APO2L)

To Page No. \_\_\_\_\_

192. The entry above is a scatter plot and histogram (which is taped to page 43) of the data I collected using FACS to evaluate the percentage of apoptotic HeLa cells from the experiment described on this page. These entries reflect the FACS data from sample 18 (HeLa cells pre-incubated with ALLN and Apo-2L), as reflected by “Sample ID:18” noted below the histogram. The data reflect the cells that were treated with ALLN or cyclohexamide in the presence of Apo-2L were apoptotic.



193. The entry above (which is taped to page 43) shows the FACS data from sample 1 (HeLa cells alone) as reflected by “Sample ID:1” noted below the histogram. The data reflect the cells that were apoptotic, which was a small percentage compared to sample #18.

194. At the bottom of this page, I noted that, "Those plates were really heavy and each individual cell may have seen less drug than usual (meaning less Apo2L)."

**Page 51, Notebook 27250 (ADE-23)**

195. I conducted the activities described on page 51 of Notebook 27250 on May 1 and 2, 1997. I recorded the activities described on page 51 of Notebook 27250 on May 2, 1997. I signed and dated the bottom of page 51 on May 5, 1997. This experiment is similar to the experiment I conducted and described on page 43 of Notebook 27250.

196. My entries reflect that on May 1, 1997, I plated 200K HeLa cells in 6 well plates.

197. My entries reflect that on May 2, 1997, I pretreated the cells with ALLN or cyclohexamide for 1 hour prior to Apo-2L. After a 5 hour incubation with Apo-2L, I harvested the cells and conducted the annexin FACS assay to determine whether the cells were apoptotic.

198. My handwritten notation at the end of each row reflects the data from the FACS analyses I conducted on the HeLa cells and indicates the percentage of cells that underwent apoptosis in response to the treatment with Apo-2L.

199. Samples 1 through 3 show the percentage of apoptosis in transfected HeLa cells without Apo-2L (NT). Samples 4 through 6 show the percentage of apoptosis in HeLa cells pre-incubated with ALLN without Apo-2L (NT). Samples 7 through 9 show the percentage of apoptosis in HeLa cells pre-incubated with cyclohexamide without Apo-2L (NT). Samples 10 through 12 show the percentage of Apo-2L-induced apoptosis in HeLa cells incubated with Apo-2L alone. Samples 13 through 15 show the percentage of Apo-2L-induced apoptosis in HeLa cells pre-incubated with cyclohexamide and Apo-2L. Samples 16 through 18 show the percentage of Apo-2L-induced apoptosis in HeLa cells pre-incubated with ALLN and Apo-2L. The data reflect that cyclohexamide and ALLN increased the level of Apo-2-L-induced apoptosis

in HeLa cells.

200. I taped images of two histograms (across the right side of page 51) of the data I collected using FACS to evaluate the percentage of apoptotic HeLa cells from the experiment described on page 51.

201. The histogram on the bottom of page 51 shows FACS data from sample 10 (Apo-2L), as reflected by "Sample ID:10" noted below the histogram. The data reflect HeLa cells that were apoptotic due to Apo-2L.

### **E. DNA Ladder Experiments**

#### **Page 89, Notebook 26508 (ADE-22)**

202. I recorded and conducted the activities described on page 89 of Notebook 26508 prior to March 17, 1997. I signed and dated the bottom of the page.

203. I attached a typed protocol sheet to page 89 which contained my handwritten notes, as reflected in the redacted image below.

TITLE _____				
<b>A</b>	<b>B</b>	293 cells	<b>R</b>	<b>C</b>
1000K cells for DNA Ladder				
1. 0.89	pRK5 (20µg/ml)			No Apoptosis
2. 1.8	2-1 (10µg/ml) + pRK5 (10µg/ml)			Block Apoptosis
3. 1.47	2-1 (10µg/ml) + CRMA (10µg/ml)			Block Apoptosis
4. 1.54	2-1 (10µg/ml) + HuTRADD-DN (10µg/ml)			Apoptosis
5. 0.98	2-1 (10µg/ml) + HuTRAF2-DN (10µg/ml)			Apoptosis
6. 0.97	2-1 (10µg/ml) + HuFADD-DN (10µg/ml)			Apoptosis
7. 0.86	2-1 (10µg/ml) + MuFADD-DN (10µg/ml)			Apoptosis
8. 1.04	2-1 (10µg/ml) + pRK5 (10µg/ml)			+ DEVD-Fmk (3µg/ml) NO Apoptosis
9. 0.68	pRKCMA(10µg/ml) + pRK5(10µg/ml)			NO Apoptosis

Add the DEVD-Fmk when the medium is changed.

204. The image above reflects that I transfected 1 million ("1000K") 293 cells to

conduct a DNA ladder experiment. During the apoptosis process, caspase-activated endonucleases cleave chromosomal DNA between the nucleosomes, generating a series of DNA fragments. These fragments form a “ladder” when the extracted DNA is separated by agarose gel electrophoresis. DNA laddering was a well known indicator of apoptotic cell death. The purpose of this experiment was to evaluate whether cells transfected with the construct 2-1 could induce DNA laddering alone or in the presence of caspase inhibitors, such as CRMA, and other adaptor proteins, such as the human dominant negative mutant of FADD (HuFADD-DN), murine dominant negative mutant of FADD (MuFADD-DN), human dominant negative mutant of TRAF2 (HuTRAF2-DN) and human dominant negative mutant of TRADD (HuTRADD-DN).

205. After performing the transfection, I took photographs of the plates and visualized the cells on the plates which exhibited membrane blebbing and lifting from the monolayer on the plate. I then extracted the DNA from the cells in each culture plate, as described on page 91 of this notebook.

206. The column under arrow “A” reflects plate numbers 1 through 9.

207. My handwritten entries under arrow “B” reflect the stock concentration of DNA in each sample plate.

208. My handwritten notations under arrow “C” reflect my microscope observations from the experiments. I attached and labeled photographs of the cells to page 89 corresponding to each sample number (#1 through #7 and #9). I did not prepare sample #8.

209. My entries reflect that: the cells on plate #1 (pRK5) were not apoptotic; the cells on plate #2 (2-1 construct and pRK5) were apoptotic; the cells on plate #3 (2-1 construct and CRMA) were not apoptotic; the cells on plate #4 (2-1 construct and HuTRADD-DN) were apoptotic; the cells on plate #5 (2-1 construct and HuTRAF2-DN) were apoptotic; the cells on

plate #6 (2-1 construct and HuFADD-DN) were not apoptotic; the cells on plate #7 (2-1 construct and MuFADD-DN) were apoptotic; the cells on plate #8 were not analyzed; and the cells on plate #9 (pRKCRMA and pRK5) were not apoptotic.

210. Toward the bottom of page 89, I made an entry noting that “20% of the cells recovered were used to prepare the apoptotic DNA” for subsequent use to evaluate the laddering of the DNA from the apoptotic cells. The DNA laddering experiment is continued on pages 91 and 93 of Notebook 26508.

**Page 91, Notebook 26508 (ADE-22)**

211. I recorded and conducted the activities described on page 91 of Notebook 26508 prior to March 17, 1997.

212. I noted at the top of page 91 that the title of this experiment was “DNA Ladder From Pg 89.”

213. My entries reflect that I used 20% of the transfected cells recovered from the experiment performed on page 89 to perform the DNA ladder experiment on this page. The purpose of this experiment was to determine if there was degradation of chromosomal DNA of the transfected cells, thus indicating apoptosis. I harvested the cells from the plates and extracted the DNA, as described below.

214. Across the middle of page 91, I made an entry noting that I extracted DNA fragments from the transfected cells using the Trevigen DNA Ladder kit, used for isolating and labeling DNA. I included one extra chloroform extraction. I labeled the DNA using  $P^{32}$  and ran the samples on a gel.

215. Across the middle of page 91, I made entries detailing how I labeled the DNA and prepared the samples for gel electrophoresis.

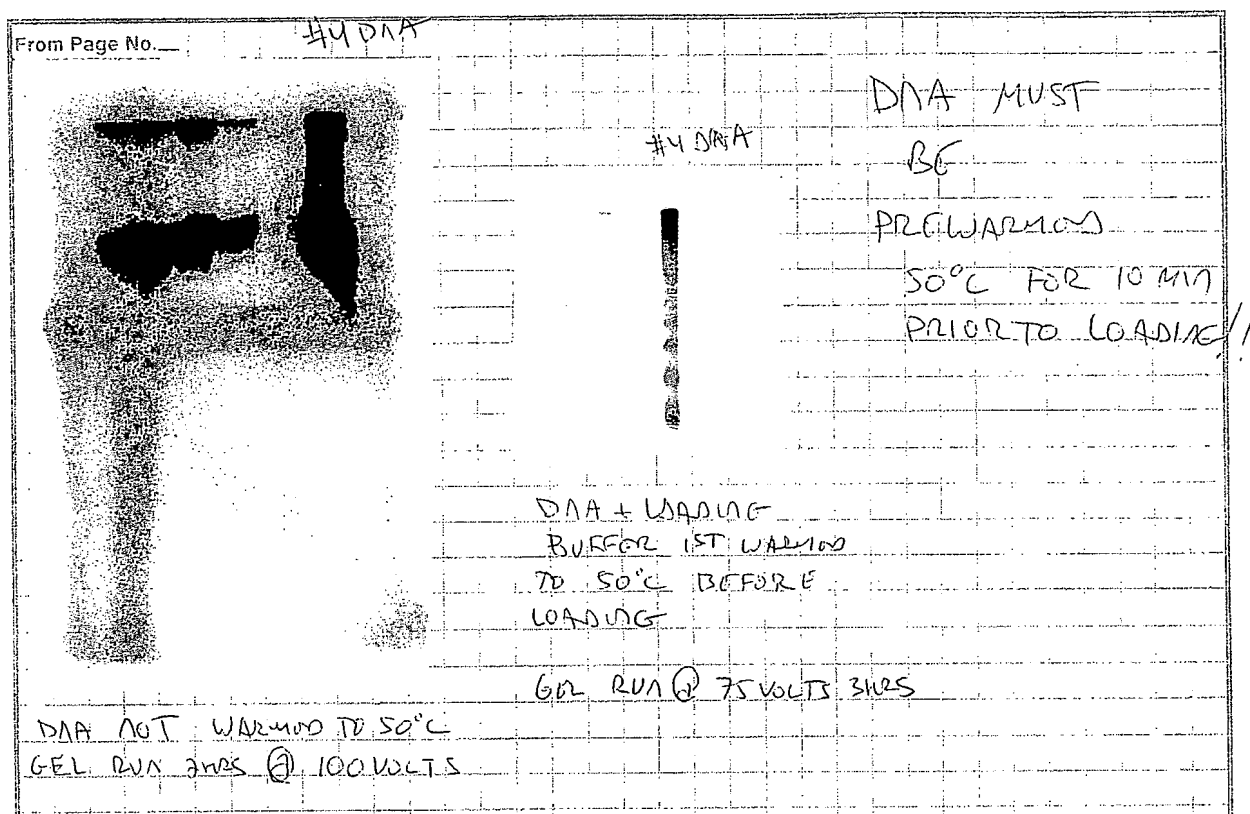
216. My entries reflect that I ran the gels for 2 hours at 100 volts. Photographs of the two gels were taped onto page 93 of this notebook.

**Page 93, Notebook 26508 (ADE-22)**

217. I recorded and conducted the activities described on page 93 of Notebook 26508 prior to March 17, 1997.

218. I made a notation at the top of page 93 that the title of this experiment was "DNA Ladder From Pg 89."

219. I made the following entry on page 93 in which I taped two photographs of gels and added my handwritten notes. This experiment was a continuation of the DNA ladder experiment I started on page 89 and continued on page 91 of Notebook 26508.



220. My entries reflect that I labeled excess DNA as described on page 91 and ran two gels. The entry above shows the two photographs of the gels which I taped onto this page. I



noted the lane that contained the “#4 DNA” in each gel. “#4 DNA” was the sample containing construct 2-1 and Hu-TRADD-DN.

221. My entries reflect that I noted under the gel on the left side of the page that, “DNA not warmed to 50°C. Gel run 2 hrs @ 100 volts.” This gel did not show a DNA ladder (indicating DNA fragments). The DNA in this gel was stuck in the wells, so I believed that the DNA needed to be pre-warmed.

222. My entries reflect that under the gel on the right side of the page, I noted that, “DNA + loading buffer 1<sup>st</sup> warmed to 50°C before loading. Gel run @ 75 volts 3 hrs.” A clear apoptotic pattern (DNA ladder) was visible from sample #4 (construct 2-1 and Hu-TRADD-DN).

**Page 1, Notebook 27250 (ADE-23)**

223. I recorded and conducted the activities described on page 1 of Notebook 27250 on March 17, 1997. This was a similar experiment to the experiment I conducted and described on page 89 of Notebook 26508.

224. I taped a protocol sheet on page 1 reflecting that I transfected 293 cells to obtain DNA for a DNA ladder experiment. The purpose of this experiment was to evaluate whether cells transfected with the 2-1 construct could induce DNA laddering alone or in the presence of caspase inhibitors and other adaptor proteins.

225. My entries reflect that in addition to the adaptor proteins I co-transfected which were described on page 89 of Notebook 26508, I also co-transfected some 293 cells with RIP-DN.

226. My entries reflect that I extracted DNA fragments from the transfected cells using the Trevigen kit, labeled the DNA using P<sup>32</sup> and ran the samples on a gel.

227. My entries reflect that I ran two gels. The first gel contained samples 1, 2, 3 and

8. The second gel contained samples 4, 5, 6, 7, 10 and 3.

228. I did not attach any photographs of the gels related to this experiment in my notebook.

**Page 6, Notebook 27250 (ADE-23)**

229. I recorded and conducted the activities described on page 6 of Notebook 27250 on March 21, 1997.

230. My entries reflect that at the top of this page was my notation, "Cleaned Up Ladder DNA From Pg. 1."

231. My entries reflect that I chloroform extracted and reprecipitated the DNA obtained from the laddering experiment described on page 1 of Notebook 27250. I washed the DNA in 70% ethanol and resuspended. I performed the DNA ladder protocol as described on page 1 of Notebook 27250. I prepared 10 samples. I added DNA from the transfected cells, H<sub>2</sub>O, 10X Klenow buffer, P<sup>32</sup> diluted 1:20 and Klenow enzyme to each tube. I ran the samples on two gels.

232. I did not attach any photographs of the gels related to this experiment in my notebook.

**Page 12, Notebook 27250 (ADE-23)**

233. I recorded and conducted the activities described on page 12 of Notebook 27250 on March 24, 1997.

234. I taped a protocol on the bottom half of page 12 pertaining to the Trevigen Ladder Kit. I also taped a photograph of a gel, as reflected in the image below. I used the "cleaned up" DNA obtained from the experiment described on page 6.

SMUPD 95-15 3-27-97 3.56n



#### Notes on Trevigen DNA Ladder Kits

**Additional extraction-** you must include a phenol/choloroform extraction followed by a chloroform extraction between steps 7 and 8.

**Thick Agarose Gel-** you must use at least 75mls in pouring your mini agarose gel (0.9cm).

**Warm the Samples-** you must warm the labeled DNA containing loading dye to 50°C for 5 min prior to loading on the gel.

-use 1µg of DNA in your reactions.

-use 1.5% Trevicil agarose.

-run 3hrs at 75Volts (cut off the gel below the bromophenol blue).

-do not fix the gel, rinse 5min in TAE buffer and dry 2hrs at 60°C.

235. My entries above reflect my notes on the Trevigen DNA Ladder Kit. A gel is shown on this page which was viewed on a phosphoimager. The samples ran on this gel are described on page 1 of Notebook 27250. Sample 1 is pRK5 vector alone. Sample 2 is Incyte construct 2-1 and pRK5. A DNA ladder is visible from sample 2. Sample 3 is Incyte construct 2-1 with CRMA. Sample 6 is Incyte construct "2-1" with HuFADD-DN. Sample 8 is Incyte construct 2-1 with pRK5. Sample 9 is pRKCRMA with pRK5.

#### Page 13, Notebook 27250 (ADE-23)

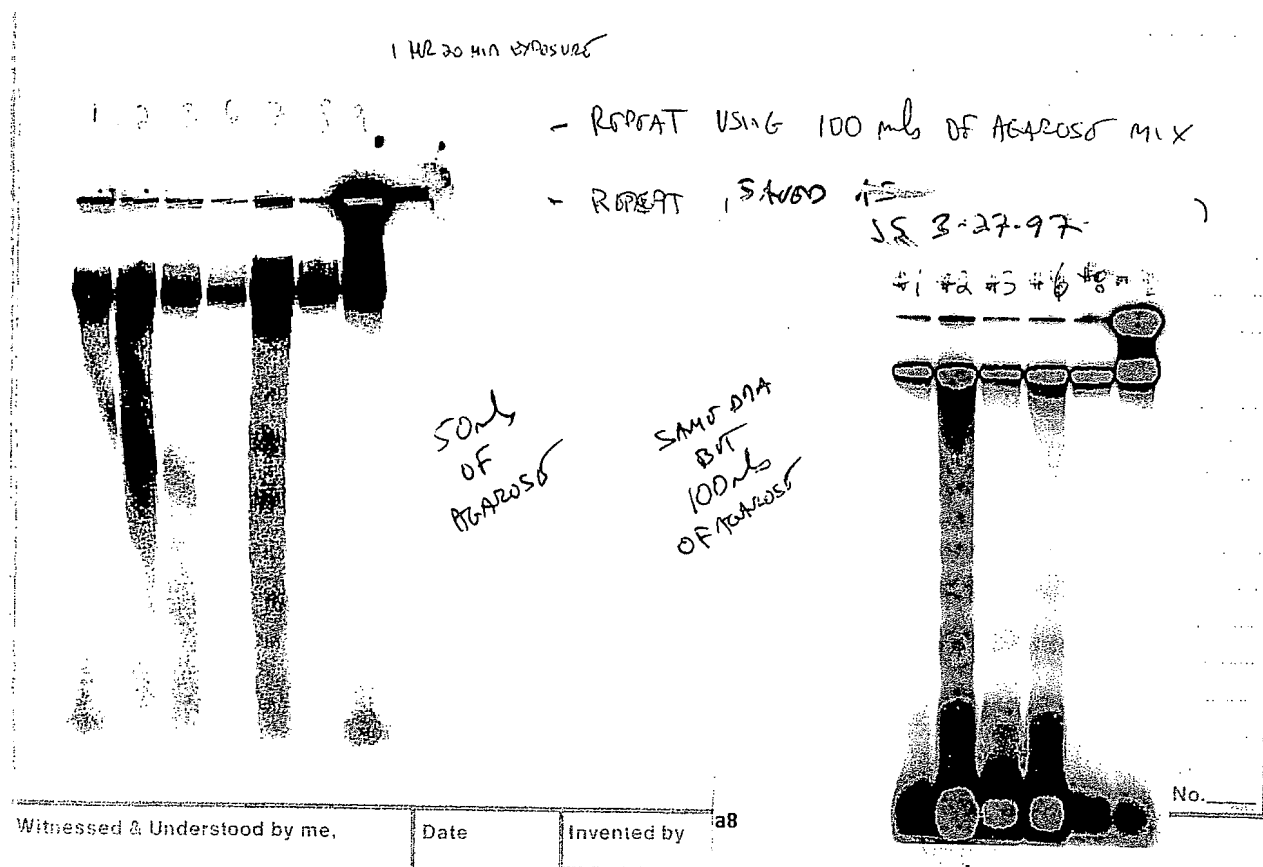
236. I recorded and conducted the activities described on page 13 of Notebook 27250 on March 24, 1997. This experiment is similar to the experiment that I conducted and described on page 1 of Notebook 27250.

237. My entries reflect that I performed a DNA ladder experiment as described on page 1 of Notebook 27250.

238. My entries reflect that I extracted DNA fragments from the transfected cells using the Trevigen protocol described on pages 1 and 12 of Notebook 27250.

239. I attached two photographs on page 13 of two gels, as reflected by the image below. My entries reflect that I analyzed the DNA fragments by gel electrophoresis. I ran one gel using 50 ml of agarose mix. This gel is shown on the left. The first gel contained samples 1, 2, 3, 6, 7, 8 and 9. Sample 2 (2-1 construct with pRK5) and sample 7 (2-1 construct with MuFADD-DN) exhibited DNA ladders, indicating apoptosis.

240. My entries reflect that I repeated the gel using 100 ml of agarose mix. The second gel contained samples 1, 2, 3, 6, 8 and 9. This gel shows that sample 2 exhibited a DNA ladder. Sample 6 (2-1 construct with HuFADD-DN) may have exhibited a DNA ladder.

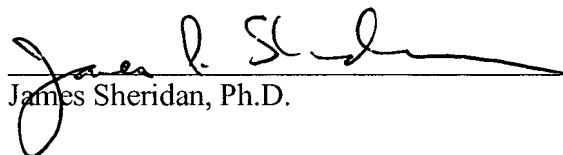


## V. Conclusion

241. I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true and further that

these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of any patents issuing from the above-identified application.

Date: 19 April 2007

  
James Sheridan, Ph.D.

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re application of:

CAMELLA W. ADAMS *et al.*

Application No. 10/052,798

Filed: November 2, 2001

For: Apo-2 RECEPTOR

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)  
) Declaration in Support of Request for  
) Declaration of Interference  
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) **Expedited Handling Requested**  
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COMMISSIONER FOR PATENTS  
P.O. Box 1450  
Alexandria, VA 22313-1450

**DECLARATION OF SCOT MARSTERS**

**ADE- 6**  
USSN 10/052,798

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## **DECLARATION OF SCOT MARSTERS**

I, Scot Marsters, declare and state as follows:

### **I. Introduction and Background**

1. I am a citizen of the United States and presently reside in San Carlos, CA.
2. I received my Bachelor's of Science degree in Microbiology and Biochemistry from the University of Maine (Orono).
3. I have been employed by Genentech Inc., South San Francisco, CA ("Genentech") since 1985.
4. I continue to be employed by Genentech and presently work as a Principle Research Associate in the Department of Molecular Oncology in the laboratory of Dr. Avi Ashkenazi. Dr. Ashkenazi was my supervisor during 1996-1998.
5. In preparation of this declaration I reviewed my laboratory notebooks 26865, 26119, and 27505 (**ADE-24, ADE-25, and ADE-26**); Maya Skubatch's notebooks 26577 and 27236 (**ADE-27 and ADE-28**); and Lakshmi Ramakrishnan's notebook 26466 (**ADE-29**). In providing descriptions of the activities recorded in the laboratory notebooks, I refer to pages in my laboratory notebooks and pages in the notebooks of Lakshmi Ramakrishnan and Maya Skubatch.
6. The descriptions I provide in this declaration include images from portions of laboratory notebook pages. The notebook pages are reproduced in their entirety in the exhibits submitted herewith. The images embedded in this declaration are accurate reproductions of the notebook pages and are intended to help guide the reader in considering my notebook entries.
7. This declaration contains a detailed description of the activities that I, or those under my supervision (such as Maya Skubatch), conducted at the direction of Dr. Ashkenazi. I am familiar with Ms. Skubatch's notebooks and recognize her handwriting in her notebooks.

This declaration also contains a detailed description of the activities conducted by Lakshmi Ramakrishnan, a scientist with whom I collaborated to isolate positive cDNA clones containing the newly identified receptor DNA from a cDNA library. I am familiar with Ms. Ramakrishnan's notebook 26466 and recognize her handwriting in her notebook. I have first-hand knowledge of the work that Ms. Ramakrishnan conducted and I reviewed her relevant notebook in preparation of this declaration. This declaration also contains descriptions of her work.

8. I understand that Genentech intends to file this declaration at the United States Patent and Trademark Office to establish activities that occurred prior to March 17, 1997, and up to May 15, 1997. I understand that March 17, 1997, is a date by which another company filed a patent application relating to the Apo-2 receptor (also known as DR5) and that May 15, 1997, is the date on which Genentech filed a patent application relating to the Apo-2 receptor. Dates preceding March 17, 1997 on documents cited in this declaration have been redacted.

9. In this declaration, I provide the following:

- a) A brief overview of the types of experiments that I, and those that I worked with, conducted while working for Dr. Ashkenazi; and
- b) A description of my activities, activities conducted on my behalf by Ms. Skubatch, and work conducted by Ms. Ramakrishnan.

## **II. Overview of Personnel and Work Conducted Involving the Apo-2 Receptor**

10. In the course of my work during 1996-1998, I routinely interacted with several Genentech scientists in the Ashkenazi laboratory including: Dr. Ashkenazi, Maya Skubatch, and Dr. James Sheridan. I routinely discussed my work and experimental results with these scientists during our interactions in the Ashkenazi lab. These scientists routinely discussed their results and activities with me as well. As I described above, I also collaborated with Ms. Ramakrishnan

to isolate positive cDNA clones containing the DNA encoding a new receptor identified by Dr. Ashkenazi.

11. Ms. Skubatch was a temporary research associate in the Ashkenazi lab. All of her work relating to the Apo-2 receptor was conducted at my request and under my direct supervision. I provide a description of her laboratory activities in a separate section of this declaration.

12. Prior to March 17, 1997, I understood that Dr. Ashkenazi was searching for receptor molecules in the Tumor Necrosis Factor (TNF) family of receptors. I understood that Dr. Ashkenazi was searching for novel receptor sequences based on structural similarities to previously identified receptors in the TNF family (*e.g.*, Apo-3 and Fas), including sequences for 1) cysteine-rich regions in the extracellular domain, 2) a transmembrane domain; and 3) an intracellular region containing a death domain.

13. My recollection is that Dr. William Wood of the Molecular Biology group at Genentech was the person most directly involved with making the Incyte sequence database information available to Dr. Ashkenazi.

14. I recall that prior to March 17, 1997, Dr. Ashkenazi searched an Incyte sequence database and identified DNA sequences that he considered to be structurally similar to the sequence of the Apo-3 receptor.

15. Prior to March 17, 1997, I clearly understood Dr. Ashkenazi's objectives regarding the newly identified receptor molecules. Dr. Ashkenazi and I often discussed the progress of my research and his research objectives. At that time, I understood that upon identifying a novel receptor sequence, Dr. Ashkenazi planned to conduct cloning and expression experiments as well as prepare a fusion protein (Apo-2-IgG (immunoglobulin G)) to use in

binding studies. Additionally, I understood that Dr. Ashkenazi planned to initiate experiments to prepare antibodies against the novel receptor, including the preparation of agonist antibodies that would specifically bind to the extracellular domain (amino acids 54 to 182) of the newly identified receptor, and that would induce apoptosis in a DR5-expressing cell.

16. Specifically, prior to March 17, 1997, I understood that Dr. Ashkenazi had contemplated a method of inducing apoptosis in a DR5-expressing cell which involved exposing the cell to an effective amount of an Apo-2 agonist monoclonal antibody which (a) binds to a soluble extracellular domain sequence (amino acids 54 to 182) of an Apo-2 polypeptide and (b) induces apoptosis in a DR5-expressing cell.

17. Once Dr. Ashkenazi identified sequences in the Incyte database, he asked me to isolate a cDNA clone having the complete sequence of the gene he identified searching the Incyte database. My activities directed to isolating clones included designing primers for use in polymerase chain reaction ("PCR") experiments, as well as designing a longer DNA probe for use in isolating the full length gene from cDNA libraries. I submitted an order form to the Genentech Synthetic Oligonucleotide facility describing the PCR primers and DNA probe which is submitted herewith as **ADE-30**.

18. I designed the primers and designated them "DD" primers. I designed a probe and designated it as the "DD" probe. I asked Lakshmi Ramakrishnan to screen various cDNA libraries and isolate clones having structural similarity to the sequences Dr. Ashkenazi identified.

19. Ms. Ramakrishnan isolated four positive clones from a pancreas cDNA library and provided them to me immediately after identifying those clones. The clones were positive because the DNA inserts in the clones hybridized to a 70-mer probe I designed to screen for the newly identified sequence. She designated the clones DDP1.1, DDP2.1, DDP3.1 and DDP4.1.

The DDP2.1 clone contained the DNA encoding the Apo-2 receptor.

20. The nomenclature of the “DDP2.1” clone evolved over time. The DDP2.1 clone was often referred to by those working on the Apo-2 receptor project as “DD.2”, “DD2”, “DD2-1” and/or “2-1.” Ultimately, once it was determined that the receptor encoded by the DD.2 DNA bound Apo-2 ligand (Apo-2L), the nomenclature shifted to “Apo-2” receptor.

21. I used the “DD” clones Ms. Ramakrishnan isolated to transform cells and overexpress the DNA contained in the clones to examine whether the DNA contained in the clones could induce apoptosis in the cells. I conducted these experiments and had assistance with the apoptosis assays from Dr. James Sheridan, a post-doctoral fellow working in Dr. Ashkenazi’s lab.

22. The transformation experiments I conducted revealed that the clone designated DD2.1 induced apoptosis in the cells. I conveyed these results immediately to Dr. Ashkenazi.

23. Once Dr. Sheridan and I observed the apoptosis-inducing effect of the DD2.1 clone and reported our results to Dr. Ashkenazi, Dr. Ashkenazi decided that we should conduct additional experiments to further characterize the DD2.1 clone.

24. The experiments that I and others conducted included making and purifying Apo-2 receptor/IgG fusion proteins. The fusion proteins were ultimately used to raise monoclonal agonist antibodies that specifically bound to the extracellular domain (“ECD”) (amino acids 54 to 182) of the Apo-2 receptor, and that induced apoptosis in a DR5-expressing cell.

25. In addition to conducting DD2.1 overexpression experiments and making fusion proteins, I, or those with whom I worked in the Ashkenazi laboratory, conducted experiments relating to the Apo-2 receptor including: 1) obtaining the complete DNA and amino acid sequence of the DD2.1 clone; 2) determining whether the Apo-2 receptor activated the NFκB

pathway; 3) evaluating expression of the Apo-2 receptor mRNA by Northern blotting experiments; 4) constructing additional fusion proteins using a FLAG epitope tag for co-precipitation studies, binding studies, and/or purification studies; 5) making GST-Apo-2 fusion proteins to search for molecules that bound to the death domain; and 6) determining the chromosomal location of the gene encoding the Apo-2 receptor.

26. Maya Skubatch, a temporary research associate in Dr. Ashkenazi's lab, assisted me with sequencing experiments, chromosomal mapping experiments, and construction of fusion proteins. Her work is described in her notebooks and I describe some of her specific laboratory activities in a later section of this declaration.

### **III. Detailed Description of Activities Reflected in Laboratory Notebook Entries and Related Documents**

#### **A. Initial Screening Work of cDNA Libraries**

27. Prior to March 17, 1997, Dr. Ashkenazi conveyed to me that he searched the Incyte database and identified novel DNA sequences having structural similarity to a portion of the Apo-3 receptor. Dr. Ashkenazi conveyed this information to me within twenty-four hours of making this discovery. Dr. Ashkenazi created a consensus sequence from the Incyte clones he identified and provided it to me.

28. I studied the sequence Dr. Ashkenazi provided to me and designed the PCR primers and a DNA probe as reflected in **ADE-30**, a request form I used to obtain oligonucleotide probes from the Genentech Synthetic Oligonucleotide facility. A redacted image of a portion of the request form is set forth below.

**Marsters, Scot** # R

REQUESTOR	cost center	phone	lab#	project #	required date	5683
Marsters, Scot	424	5747	10230	1110	R	

FRAGMENTS	O.D.	Sequence	Special Instructions
dd.probe 70 mer	1	GGG AGC CGC TCA TGA GGA AGT TGG GCC TCA TGG ACA ATG AGA TAA AGG TGG CTA AAG CTG AGG CAG CGG G	DNA reference:
dd.pcr.5' 24 mer	1	GGG AGC CGC TCA TGA GGA AGT TGG	DNA reference:
dd.pcr.3' 24 mer	1	GGC AAG TCT CTC TCG CAG CGT CTC	DNA reference:
mer			reference:

C
B
A

29. Under the “Requestor” entry on the oligonucleotide request form is my name, reflecting that I submitted the form.

30. The entry highlighted by the arrow “A” is “dd.probe” and has the designation “70 mer” below the dd.probe entry. These entries reflect that I named the probe “dd.probe” and that it was 70 oligonucleotides in length. The sequence of the probe I requested is provided immediately to the right of the “dd.probe” and “70 mer” designations in the image above.

31. The 70-mer probe was radiolabeled and used as a tool to visualize (by autoradiography) the binding of the probe to complementary sequences obtained from a cDNA library.

32. The entry highlighted by the arrow “B” in the image above is “dd.pcr.5’” and has the designation “24-mer.” These entries reflect that this 24-base oligonucleotide was used as a PCR primer and that it corresponds to the 5’ upstream sequence of the consensus sequence that Dr. Ashkenazi provided to me. The sequence of the 5’ dd primer is set forth to the right of the dd.pcr.5’ designation.

33. The entry highlighted by the arrow “C” is “dd.pcr.3’” and has the designation

“24-mer.” These entries reflect that this 24-base oligonucleotide was used as a PCR primer and that it corresponds to the 3’ downstream sequence of the consensus sequence that Dr. Ashkenazi provided to me. The sequence of the 3’ dd primer is set forth to the right of the dd.pcr.3’ designation.

34. I gave the 70-mer probe to Ms. Ramakrishnan to use as a tool to identify cDNA sequences obtained from cDNA libraries that hybridized to the probe.

35. Ms. Ramakrishnan also used the PCR primers I designed to amplify the target sequences from a cDNA library. This process allowed her to “rescue” the sequences amplified by the PCR experiments from the other sequences present in the cDNA library. Following amplification, Ms. Ramakrishnan screened the cDNA for positive clones.

36. At my request, Ms. Ramakrishnan conducted the screening of a pancreas cDNA library. She identified four positive clones that hybridized to the “DD” probes that I designed. Ms. Ramakrishnan designated the clones “DDP.1.1, DDP2.1, DDP3.1 and DDP4.1.” The “DD” reflects my original designation for the PCR primers and DNA probe; the “P” reflects that Ms. Ramakrishnan isolated the clones from a pancreas cDNA library; and the numbers 1-4 reflect that she isolated four clones.

37. I describe below Ms. Ramakrishnan’s notebook pages corresponding to some of her activities directed to isolating the positive “DD” clones. I was familiar with the work conducted by Ms. Ramakrishnan and I reviewed Ms. Ramakrishnan’s notebook 26466 in preparation of this declaration. Pages 60, 71, and 73 from Ms. Ramakrishnan’s notebook 26466 reflecting her work involved with the Apo-2 receptor are submitted herewith in **ADE-29**.

38. Ms. Ramakrishnan conducted and recorded the activities described on pages 60, 71, and 73 of her notebook 26466 prior to March 17, 1997, as reflected by the dates recorded on



those pages.

39. Across the bottom of page 60, Ms. Ramakrishnan made the following entries:

Scott - brought a Pancreas library - to make ssDNA - To screen for a novel protein - homolog of Apo-3 - has Death Domain

- Started to make ssDNA Pancreas on [R] & completed on [R]

[R] Plated  $10^6$  cells

40. The boxes containing “R” in the image above are examples of where the dates have been redacted.

41. The entries Ms. Ramakrishnan made at the bottom of page 60 reflect that I gave her a pancreas library from which she prepared single stranded DNA (ssDNA) to screen for a “novel protein-homolog of Apo-3.”

42. I recognize the handwriting at the bottom of page 71 as Ms. Ramakrishnan’s. She made the entries reflected in the image below:

Old Rescue of DD [R]

4 positives - cannot pick singles - so plate  $2^o$  screen do same as above pick into 100x & plate  $\sim 3-5 \times 10^{-2}$

A  $\rightarrow 2^o$  DD P.1  $\rightarrow$  P.4

43. These entries reflect that Ms. Ramakrishnan conducted work on an experiment she called “Old Rescue of DD.” Ms. Ramakrishnan wrote that she identified “4 positives” but that she was unable to pick single colonies (“cannot pick singles”). She plated the transformants in a secondary screen to try to obtain the positive clones.

44. The notation at arrow “A” in the image above reflects that Ms. Ramakrishnan designated the secondary screen DD clones as “DDP.1” through “DDP.4”. “ $2^o$ ” is the

designation for secondary. "DD" is the death domain designation she had been using for this series of experiments (which arose from my original probe/primer nomenclature). The "P" reflects that Ms. Ramakrishnan was working with pancreas DNA. The numbers 1 through 4 reflect that she believed that there were 4 positive transformants from the pancreas library.

45. Ms. Ramakrishnan made the following entries at the bottom of page 71:

Screen DDP1-P.4 - lift, process, prehyb & hyb for ~ 3-4 hrs			
- Wash as usual - filters quite hot! <del>quite exposure</del>			
get short exposure <del>for 1 hr</del> - picked 1 positive for each			
Witnessed & Understood by me,	Date	Invented by	Date

46. The entry reflects that Ms. Ramakrishnan lifted the filters from the secondary screens she designated "DDP1-P.4", processed the filters, prehybridized the filters, and hybridized the filters using the same radiolabeled 70-oligomer probe that she used in her previous screens. Ms. Ramakrishnan then washed the filters to remove any unbound labeled probe. She noted that the filters were "quite hot!" reflecting that a short exposure to autoradiographic film should be sufficient to produce a signal on the film.

47. Ms. Ramakrishnan noted that she "picked 1 positive for each for minipreps" reflecting that she identified positive clones and that she would conduct a DNA "miniprep" to purify the DNA from the positive clones that she picked based on the filters and transformants.

48. Ms. Ramakrishnan made the following entries at the top of page 73:

From Page No. _____	R
DD - 2 <sup>nd</sup> Screen from Pancreas	
- Picked 4 positives, 1 from each 2 <sup>nd</sup> screened & put up minipreps & also for large scale cultures for Scott/Avi's lab to put into assays	

49. These entries reflect that Ms. Ramakrishnan picked 4 positive transformants

designated DDP.1 through DDP.4, to be purified using DNA minipreps and also for large-scale cultures for me and Avi Ashkenazi to be used in assays (“...large scale cultures for Scott/Avi’s lab to put into assay.”)

50. After Ms. Ramakrishnan identified the positive clones, she immediately informed me of her results and I, and others in Dr. Ashkenazi’s lab, began work with the DD clones.

**B. Activities I Conducted Following Initial Identification of the Positive Clones**

51. I submitted the clones Ms. Ramakrishnan isolated to the Genentech DNA sequencing facility within a day or two of receiving the clones.

52. Prior to March 17, 1997, I obtained the complete sequence of the DD.2 clone from the Genentech Sequencing Facility as reflected in **ADE-36**.

53. Following Ms. Ramakrishnan’s identification of the positive clones, I conducted a variety of experiments to characterize the DD2 DNA sequence and protein. I made a record of these activities in my laboratory notebooks.

54. I maintained three separate notebooks which included descriptions of my activities directed to the Apo-2 receptor project.

55. I labeled Notebooks 26865 and 27505 as “Cell Culture” notebooks because many of the experiments described in those books primarily (but not exclusively) involved cell culture experiments. I labeled notebook 26119 as “Mol Bio” reflecting that was a notebook wherein I recorded my experiments primarily involving molecular biology. I labeled the notebooks on their respective spines.

56. The relevant pages from the notebooks are submitted as exhibits **ADE-24**, **ADE-25**, and **ADE-26**. I make reference to the page numbers from each respective notebook in the descriptions of my laboratory activities provided herein.

C. Activities I Conducted and Recorded in My Notebooks

1. Page 47, Notebook 26865 (ADE-24)

57. I conducted and recorded the activities described on page 47 prior to March 17, 1997, as reflected by the date recorded on that page.

58. The entries I made on page 47 reflect that I conducted cell transfection experiments using 293 cells and various DNA constructs. 293 cells are commercially available and derived from a human embryonic kidney cell line.

59. The purpose of the cell transfection experiments was to introduce DNA into the 293 cells, obtain expression of the protein encoded by the DNA in the cells, and observe whether the expression could induce apoptosis in the target cells.

60. Across the top of page 47, I made the following entries:

R	Set up Tx of 293 cells. 100mm Dishes.
	Fed cells 8:00am
Tube A	Tube B
0.5 ml's 1:10 TE	0.5 ml's 2x HBS
50 µl 2.5M CaCl <sub>2</sub>	
1 µl VAPUA	
10 µg DNA Below	

61. These entries reflect that I set up transfection experiments using 293 cells and prepared two tubes (A and B) containing the reagents I used for the calcium phosphate transfection of the 293 cells I used in this experiment. Tube A included ten micrograms of the specific DNA I used to transfect the 293 cells on each plate (e.g., plate 15 containing DNA designated DD.2.1).

62. The relevant plate numbers using DD.2.1 DNA, and a description of the various other DNA I used to transfect the cells on each plate, are reflected in the following entries:

Plate # DNAs

14	DD 1.1	} Incyte
15	" 2.1	
16	" 3.1	
17	" 4.1	

2078364  
1237537

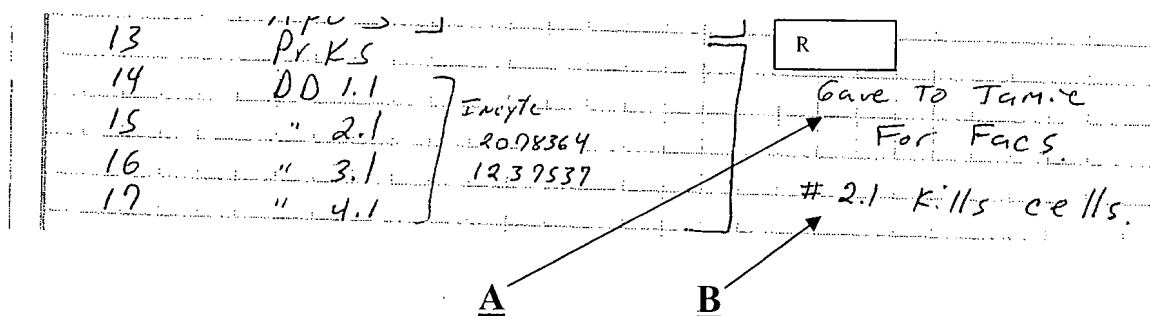
63. My entries reflect that plates 14 through 17 bear a common "DD" designation followed by "1.1," "2.1," "3.1," and "4.1," respectively. I used these clones to transfect the 293 cells.

64. As described above, the clone designations were made by Ms. Ramakrishnan and were a result of her work directed to screening a pancreas cDNA library and identifying four positive clones that hybridized to the 70-mer DD probe that I designed.

65. I made a bracket encompassing plates 14-17 which is followed by the word "Incyte." Below the term "Incyte" I listed the numbers 2078364 and 1237537. These numbers reflected the Incyte clone numbers that provided the sequence information from which Dr. Ashkenazi designed a consensus sequence which I, in turn, used to design the PCR primers and DNA probes that Ms. Ramakrishnan used to isolate the four positive "DD" clones.

66. Plate 15 bears the designation DD2.1 which reflects that DNA having the DD2.1 designation was used to transfect the cells on plate number 15. I received the DD.2.1 DNA construct from Ms. Ramakrishnan. DD.2.1 was later designated Apo-2 receptor based on the fact that overexpression of the DD.2.1 DNA caused apoptosis in the cells and that it was confirmed to encode a receptor that bound Apo-2 ligand.

67. I made the following entries toward the bottom of page 47:



68. The notation at arrow "A" reflects that I gave the cells transfected in plates 13, 14, 15, 16, and 17 to "Jamie" for FACS (fluorescence activated cell sorter) analysis. "Jamie" is Dr. James Sheridan, a scientist employed by Genentech at the time I conducted these experiments, who routinely conducted FACS analyses.

69. FACS analyses were routinely used by Dr. Sheridan to measure the fluorescence associated with Annexin V binding to phosphatidylserine on cells that were undergoing apoptosis.

70. The notation at arrow "B" reflects that the clone number designated 2.1 "kills cells." The "#2.1" designation corresponds to the DD2.1 DNA I used to transfect the cells on plate 15. I recall looking at the transfected cells under a microscope and believing that the cells were undergoing apoptosis based on the morphological changes that occurred in the cells. Dr. Sheridan confirmed that the DD2.1 clone induced apoptosis in the cells using the FACS analyses. I conveyed these results to Dr. Ashkenazi and showed him the apoptotic and control cells.

71. Dr. Sheridan took photographs of the cells undergoing apoptosis following overexpression of the DD2.1 DNA and showed them to me and Dr. Ashkenazi.

72. This series of experiments confirmed that the DD2.1 DNA induced apoptosis when overexpressed in 293 cells.

## 2. Page 41, Notebook 26119 (ADE-25)

73. I conducted and recorded the activities described on page 41 prior to March 17, 1997, as reflected by the dates recorded on that page.

74. I wrote "Make New Death Domain Receptor IgG" across the top of page 41 reflecting that the experiments I conducted and recorded were directed to making a receptor IgG fusion protein with the new Death Domain receptor (*i.e.*, DD.2.1 or Apo-2).

75. As reflected by my notebook entries, I set up a PCR reaction using DD2.1 DNA and 5' and 3' primers directed to portions of the pRK5 vector harboring the DD2.1 DNA insert (prkrev), and other primers designated "SM.1", "SM.2", and "SM.3". The sequence of the SM.1, SM.2, and SM.3 primers is set forth in ADE-31. These activities are reflected at numbers one through three on the right side of page 41, an image of which is set forth below.

Make New Death Domain Receptor IgG

Project No. \_\_\_\_\_  
Book No. 26119

41

TITLE \_\_\_\_\_

From Page No. \_\_\_\_\_

R Set up PCR's

	DNH	Primer	5'	3'
0.5 µL DNA				
10 µL 10x PCR Buffer				
24 µL 125 mM dNTPs				
1 µL 5' primer	1) DD 2.1	prkrev		SM.3
1 µL 3' primer	2) "	SM.1		"
63 µL dH <sub>2</sub> O	3) "	SM.2		"
1 µL Taq	4) DDK 4.1	prkrev		"
± 0.1	5) "	SM.1		"
	6) "	SM.2		"
3' 94°				
1 cycle				
1' 94°				
1' 50°				
3' 72°				
16 cycles				

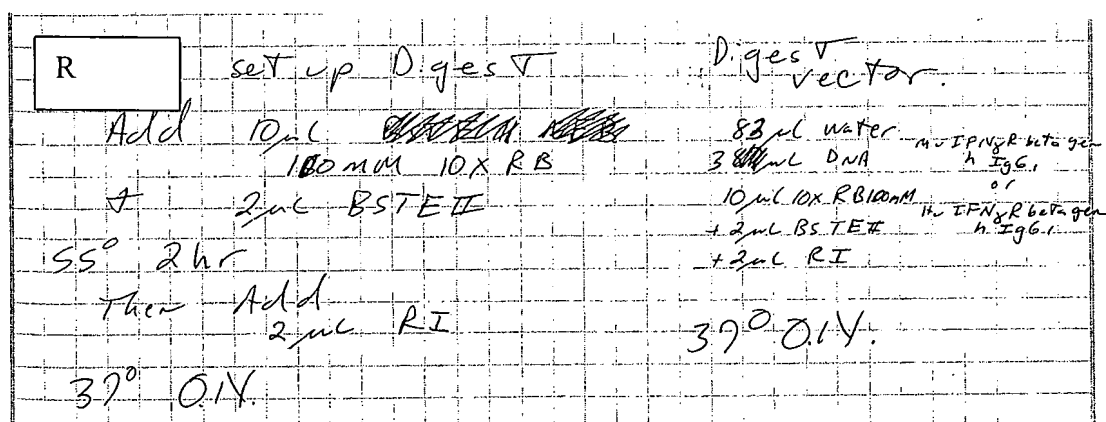
ran 0.1X

76. The additional entries on the left side of page 41 reflect the reagents I used in the

PCR experiment, the experimental conditions, and that I ran the PCR reaction overnight (*i.e.*, “ran O.N.”).

77. The goal of the PCR experiment was to amplify the DD2.1 DNA in such a way as to generate a DNA construct that could be used to create a fusion protein made up of the DD2.1 DNA and DNA encoding antibody sequences from IgG proteins.

78. Toward the bottom of page 41, my entries reflect that I continued my work toward generating a DD2.1-IgG fusion protein and digested vectors containing human and murine interferon alpha receptor IgG fusion proteins with the BstEII restriction enzyme. I made the constructs that I used in these experiments. I used the DNA encoding the IgG portion of this particular fusion protein as “donor” sequence to be ligated into vectors with the PCR products obtained from the DD2.1 PCR amplification. This work is reflected in the redacted image of page 41 below, under the notations “set up Digest” and “Digest vector.” The entries reflect that I digested two sets of vectors to later use to ligate the fusion inserts.



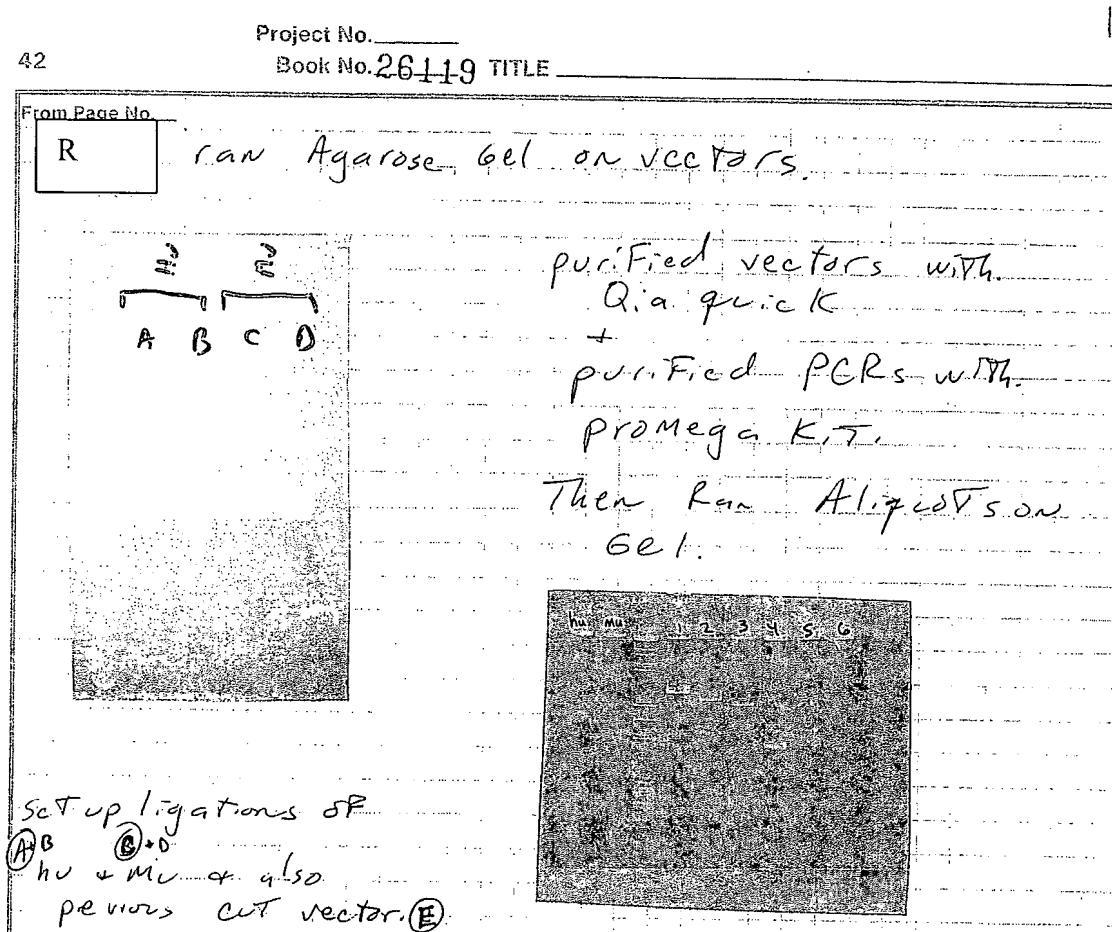
### 3. Page 42, Notebook 26119 (ADE-25)

79. I conducted and recorded the activities described on page 42 prior to March 17, 1997, as reflected by the dates recorded on that page.

80. The activities I recorded on page 42 are a continuation of my activities described



on page 41. The experiments on page 42 reflect that I "ran agarose gel on vectors" which I described on page 41, and that I purified the vectors using "Qiaquick," a commercially available purification kit from Qiagen. I purified the DD2.1 PCR fragments using a commercially available kit from Promega and ran aliquots of the purified vector and PCR fragments (1-6) on the gel. These activities are reflected in the redacted image of page 42 set forth below:

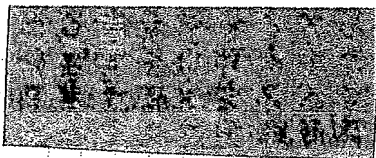


81. I wrote the "hu" and "mu" designations on the photographs of the gels taped to page 42, and on the page itself, which correspond to "human" and "murine." As described on page 41, I used human and murine interferon alpha receptor-human IgG fusions as a source of human IgG DNA sequence to ligate to the DD2.1 sequence obtained from the PCR amplification

reactions.

82. The numbers one through six listed on the photograph of the gel on the right side of the page correspond to the PCR products I obtained from reactions one through six described at the top of page 41. Numbers one through three correspond to DD2.1 PCR products.

83. After purification and visualization of the vector and DD2.1 PCR products, I ligated the DD2.1 PCR products ("fragments 1, 2, + 3" as described on page 41) and the vectors harboring the human IgG sequence, using a "rapid ligation kit." I then transformed JM109 cells (an *E. coli* bacterial strain) using the newly ligated vector that I believed contained the DNA encoding the DD2.1-IgG fusion protein. I recorded these activities at the bottom left of page 42, an image of which is provided below.

Set up ligations of (A+B) (B+D) hu. & mu. & also previous cut vector. (E) with Fragments 1, 2 & 3 use rapid ligation K.T. 1ul vector 2ul insert 2ul vial 2 10ul vial 1 mix <del>5ul vial 3</del> 1ul vial 3 5 min RT Transformed into JM109's			
Witnessed & Understood by me,	Date	Invented by	Date
			To Page No. ____

4. Page 56, Notebook 26865 (ADE-24)

84. I conducted and recorded the activities described on page 56 prior to March 17, 1997 as reflected by the dates recorded on that page.

85. I made the following entry at the top of page 56:

Project No. \_\_\_\_\_  
Book No. 26865 TITLE \_\_\_\_\_

Northerns From Maya  
OR New DD. Receptor.  
2.1

86. I inserted images obtained from the Northern analyses in a pocket taped on page 56.

87. The entry I made on page 56 reflects that "Maya" (Skubatch) conducted Northern blot analyses to examine mRNA levels of the "New DD Receptor 2.1" in various tissue types. She recorded her activities directed to this experiment on pages 76-79 of her notebook 26577. I describe these activities in a later section of this declaration.

88. The results located in the pocket on page 56 reflect that DD.2 mRNA was detected in fetal kidney, liver, and lung, and adult peripheral blood leukocytes (PBLs), colon, small intestine, ovary, testis, prostate, thymus, spleen, pancreas, kidney, skeletal muscle, liver, lung, placenta, and heart.

**5. Page 62, Notebook 26865 (ADE-24)**

89. I conducted and recorded the activities described on page 62 prior to March 17, 1997, and on March 18, 1997, as reflected by the dates recorded on that page.

90. The purpose of the transfection experiments I conducted was to express the DNA construct encoding DD2-IgG fusion proteins in 293 cells. The fusion proteins could then be readily purified using affinity techniques. Purified fusion protein was later used as an antigen for immunization of mice to generate monoclonal antibodies.

91. I made the entries in the image below across the top of page 62 reflecting that I conducted transfections of 293 cells (in the "Title" section), and includes a brief description of

the DNA I used to transfect the cells. I noted that tubes 3-26 contained "DD.2-IgG" DNA whereas tube 1 contained vector only (as a negative control) and tube 2 contained an Apo-3-IgG fusion (as a positive control). The numerical designations next to the DD2 fusions (e.g., #20) reflect that the fusion proteins came from particular clone numbers. Maya Skubatch prepared DNA of these clones under my direction and supervision, and she recorded her activities on pages 84-85 of her notebook 26577, which I describe in a later section of this declaration.

62

Project No. 2935  
 Book No. 26865 TITLE Trans FectiOns

From Page No. R

TX OF 293 cells Fed 12:00 noon

Tube #	DNA
1	PrKS
2	Apo-3-IgG #31
3	DD.2-IgG #5
4	DD.2-IgG #20
5-15	DD.2-IgG #6
16-26	DD.2-IgG #20

DNAn cells  
# 4:00 pm

92. The redacted image below of page 62 reflects that plates 1-4 were radiolabeled with "0.3 mici" (0.3 millicuries) of ProMix, which is a commercially available reagent used for *in vitro* protein labeling with radioactive methionine and cysteine. The purpose of labeling the proteins in plates 1-4 was to incorporate a radioactive label into the proteins to allow for visualization of the proteins in assays that require measurement or detection of radiation (e.g., autoradiographic assays).

R

plates 1-4 Labeled with  
2.5 ml's Met - Cys media 8:50am  
+ 0.3 ml's Pro Mix  
535 Met + Cys

plates 5-26 Fed S.F. Media Noon

Harvest sups off plates 1-4 3:00pm

3/18/97 Harvest S.F. sup off plates

Hot sups  
pg #66

93. The redacted image further reflects that plates 5-26 were fed "S.F. media." "S.F media" is shorthand for "serum free" media and was a readily available and routinely used culture medium used to grow cells in culture.

94. I harvested the "sups," or supernatants, from plates one through four, which were radiolabeled using the Pro-mix reagent. On March 18, 1997, I harvested the supernatants off of plates 5-26 which I previously fed with serum free media. I recorded these activities toward the middle and bottom of page 62 as reflected in the image below.

95. I noted at the bottom right of page 62 that "Hot sups pg #66" which reflects that I conducted additional experiments on page 66 using the radiolabeled supernatants obtained from plates one through four described at the middle of page 62.

96. I used the labeled proteins I prepared in a precipitation experiment described on page 66.

**6. Page 65, Notebook 26865 (ADE-24)**

97. I conducted and recorded the activities described on page 65 prior to March 17, 1997, as reflected by the dates recorded on that page.

98. The purpose of these experiments was to identify the chromosomal location of the DD.2 DNA.

99. At my request and under my supervision, Maya Skubatch conducted the radiation hybrid work underlying the chromosomal mapping of the DD.2 gene. She recorded her activities on page 85 of her notebook 26577. I describe her activities in a later section of this declaration.

100. Ms. Skubatch provided me with the results of her radiation hybrid experiments using the DD.2 DNA. She used a commercially available kit to perform the mapping experiment.

101. I sent the DNA sequence information (marker) to the Stanford University Hybrid Mapping service in electronic form and asked for a chromosomal map for the DD.2 DNA sequence information I provided. This activity is reflected in the e-mail attached to page 65 of my notebook and a redacted image of a copy of the e-mail I sent to the Stanford Radiation Hybrid Mapping Server at SHGC ([rhserver@shgc.stanford.edu](mailto:rhserver@shgc.stanford.edu)) is provided below.







“Rips” experiments, as reflected by the entry at the top of the page “Setup Rips of Sups from [redacted date] pg. #62.” “Rips” is my shorthand for Radio-Immuno Precipitation. Rips experiments were used to measure the amount of protein contained in the supernatant from cells transfected with the DD2-IgG fusion protein. The experiment I described on page 66 involved using an IgG-specific binding agent to precipitate radioactive DD2-IgG fusion proteins that I described on page 62.

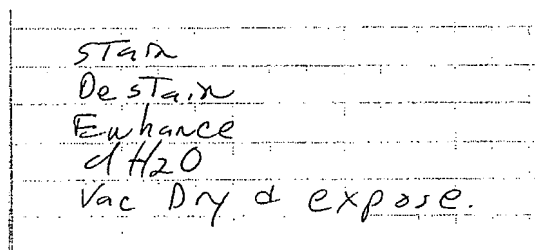
66	Project No.	Book No. 26865	TITLE
From Page No.	3/17/97 Setup Rips of Sups From		
	R	pg # 62	

108. The entries on page 66 reflect that I collected the supernatants from the cells from plates one through four described on page 62. I then incubated the supernatants in a buffer containing various reagents including detergents and “Pansorbin,” the latter of which would bind to the IgG moiety of the fusion protein.

109. After incubation, I boiled the reagents for 5 minutes to release the DD2-IgG fusion proteins bound to the Pansorbin. I then loaded the samples on a gel in lanes in the order described on page 66 under the phrase “Load gel.” My activities are reflected in the images of portions of my notebook below.

Add 15 $\mu$ l	1x sample	Buffer	NO DTT
Boil 5 min.			
Load Gel.			
/ Dye / Dye / P.K.s /	APD. 3 / IgG #6	DD. 2 / IgG #20	DD. 2 / IgG #20 / M / Dye / Dye / Dye /

110. After I loaded the gel as described above, I stained the gel with Coomassie blue (to confirm the presence of protein of the expected size), destained the gel, enhanced the gel using a commercially available kit to better visualize any banding pattern, washed the gel with distilled water, dried the gel under a vacuum, and exposed the gel to autoradiographic film. My entries in my notebook and the image of the entries reproduced at the bottom of page 66 below reflect those activities.



A photograph of a handwritten note on a piece of lined paper. The text is written in cursive and lists the following steps: "STAIN", "De STAIN", "Enhance", "d H<sub>2</sub>O", and "Vac Dry & expose." The paper is slightly aged and the handwriting is in dark ink.

111. I included the resulting images from the gel in a pocket that I taped onto page 66. One image reflects development of the gel at one hour and another reflects development of the gel after exposure overnight. The images from the gel indicate that there may have been a low level of the DD2-IgG fusion protein expressed.

#### 8. Page 67, Notebook 26865 (ADE-24)

112. I conducted and recorded the activities described on page 67 on March 18, 1997, as reflected by the dates recorded on that page.

113. At the top of page 67, I noted that "Took 1.5 mls OF sup from pg #62 cold" and listed "prk5, DD.2-IgG #6 and DD.2-IgG #20" below that entry. These entries reflect that I used non-radioactive supernatant from cultures of cells I previously transfected with vector alone (prk5) or DNA encoding DD.2-IgG fusion proteins.

114. The purpose of these experiments was to evaluate whether the cell supernatants expressed DD2-IgG fusion proteins.

115. An image of a portion of page 67 is set forth below. My entries reflect that I

added the supernatants from page 62 to protein A sepharose. The protein A was used to purify DD2-IgG fusion proteins from the supernatants by a standard technique called protein A affinity chromatography. Following incubation at room temperature (*i.e.*, "2 hr RT on rotator"), I washed the samples with buffer.

116. I then divided the samples into two sets of tubes wherein one tube set contained the reducing agent dithiothreitol (DTT) and the other tube set did not. My activities are reflected by the entry toward the middle of page 67 providing "Add sample Buffer no DTT to 1 set" and the entries directly beneath reflecting adding sample buffer "+ DTT to 1 set." I then boiled the samples for 5 minutes (*i.e.*, "Boil 5'") to release the fusion proteins from the protein A beads, and loaded the samples on a gel to resolve the proteins, in the order described under the entry "Load gel."

Project No. \_\_\_\_\_

Book No. 26865

67

From Page No. \_\_\_\_\_

3/18/97

TOOK 1.5 ml's OF sup FROM pg # 62 CONT

PKS

DD.2-IgG #6

DD.2-IgG #20

Add 30  $\mu$ L OF Protein A Sepharose

2 hr RT on ROTATOR

Wash 3X PBS

SPLIT TO TWO TUBES

Add Sample Buffer <sup>NO DTT</sup> TO 1 SET

" " " + DTT TO 1 SET

Boil 5'

Load Gel

NO DTT				+ DTT			
empty	PKS	DD.2-IgG #6	DD.2-IgG #20	empty	PKS	DD.2-IgG #6	DD.2-IgG #20
						M	empty

Transfer to nitrocellulose +

Put in ~~Block~~ SP2 M.I.E + PBST O.N.

TO BLOCK

117. After I ran the gel with the fusion samples, I transferred the samples contained on the gel to nitrocellulose paper (blot), and incubated the blot in a 5% milk solution + PBST (PBS plus Tween) overnight. The purpose of this was to block non-specific binding sites to which an antibody might bind and give a false positive.

118. On March 18, 1997, I incubated the blocked blot containing the DD2-IgG fusion samples with a murine (mouse) antibody that binds the human IgG portion of the DD2-IgG fusion protein. The commercially available murine antibody is conjugated to horseradishperoxidase (“HRP”), which is an enzyme that produces a signal when incubated with an appropriate substrate. A positive HRP signal (*i.e.*, “chemiluminescence” as I described on page 67) after washing of the blot indicates specific binding of the antibody to the IgG portion of the DD2-IgG fusion protein. These activities are reflected in my notebook entries at the bottom of page 67 and in the image below. I wrote “70” under the “To Page No. \_\_\_” entry reflecting that I recorded additional data relating to this on page 70. Page 70 contains a pocket containing the results from this experiment. The blots do not appear to reflect any positive results.

3/18/97	Add Mur-Hu-IgG	HRP (1:1000)	2 hrs
	Wash 3x	PBST	5 min
Develop By chemiluminescence			
Witnessed & Understood by me,	Date	Invented by	Date
			To Page No. 70

9. **Page 68, Notebook 26865 (ADE-24)**

119. On March 18, 1997, I filled out an assay request form and attached it to page 68 of my notebook 26865. I note that the date on page 68 reads March 18, 1996, which appears to have been an error on my part. The correct date is 1997 based on the context of the information provided in the request form.

120. A portion of the request form is provided in the image below.

From Page No. 3/18/96 Sent sups to Assay services for h<sub>u</sub> Fc elisa

ASSAY: To sign up for assays call x2632 Drop off samples in room 5295.		A.S. TECH		TEST PROCEDURE	
SAMPLES SUBMITTED BY <u>Scot Marsters</u>	EXTENSION <u>1847</u>	MAIL STOP # <u>410</u>	COST CENTER <u>424</u>	PROJECT CODE <u>1110</u>	PRODUCT ID. <u>DD2-IgG</u>
DATE SUBMITTED <u>3/18/97</u>	DATE TO BE ASSAYED <u>3/19/97</u>	<input type="checkbox"/> WILL PICK UP <input type="checkbox"/> MAIL RESULTS <input type="checkbox"/> FAX RESULTS (Log in: <u>SHM</u> ) <input type="checkbox"/> CALL ME		STORAGE TEMP. (PRE-ASSAY) <input type="checkbox"/> AMBIENT <input type="checkbox"/> -10°C OR BELOW <input type="checkbox"/> 2° - 8°C <input type="checkbox"/> OTHER	
<input type="checkbox"/> GMP <input type="checkbox"/> GLP Study No. <input type="checkbox"/> N/A	SAMPLE MATRIX <u>1110</u>	ANTICOAGULANT <u>if used</u>	<input type="checkbox"/> RADIOACTIVE <input type="checkbox"/> BIOHAZARD (NON-HUMAN/NON-PRIMATE) Specify type and amount:	SAMPLE OF HUMAN/PRIMATE ORIGIN? <input type="checkbox"/> NO <input type="checkbox"/> YES If yes, identify source: <u>293-10/15</u> (specify tissue, cell line, blood, etc.)	
SAVE SAMPLES? <input type="checkbox"/> YES: Storage Temp. <input type="checkbox"/> NO: Samples not picked up within 2 weeks will be discarded.		NOTES:			
NOTES: <u>Please Run samples in Duplicate</u>		TIME OF REPORT:			
		DATE			
		TIME			
		INITIALS			
PLEASE CALL THE LAB WITH ANY QUESTIONS OR PROBLEMS REGARDING ASSAY RESULTS					

121. The entry across the top of the notebook page reflects that I sent supernatant samples to the Genentech assay services group and asked them to conduct an ELISA experiment to detect human Fc protein. The human Fc fragment was part of the DD2-IgG fusion protein and detection of Fc by ELISA would confirm that the DD2-IgG protein was present in the supernatants I harvested from the cells I transformed with the DD2-IgG construct.

122. The request form further reflects additional information including: 1) that I submitted the samples as reflected by "Scot Marsters" in the "Samples submitted by" box; 2) that the sample was "DD2-IgG" as reflected by the same designation in the "Product Id." box; and 3) that the samples were submitted on "3/18/97" as reflected by that entry in the "Date Submitted" box.

123. The bottom of the request form contains the raw data from the ELISA assay. The data did not reveal any positive results.

#### 10. Page 69, Notebook 26865 (ADE-24)

124. I conducted and recorded the activities described on page 69 on March 18, 1997,

as reflected by the dates recorded on that page.

125. I noted at the top of page 69 “Make NFκB probe” reflecting that I made radioactive probes for use in NFκB assays. I provided the details of the protocol I followed on page 69. I further included the raw data sheet from the scintillation counter measuring the counts per minute (cpm) of the radiolabeled NFκB probe. The estimated cpm for the probe I made was  $1.4 \times 10^6$  cpm/ul which provided sufficient radioactivity for use in my NFκB experiments.

**11. Page 71, Notebook 26865 (ADE-24)**

126. I conducted and recorded the activities described on page 71 on March 19, 1997, as reflected by the dates recorded on that page.

127. My activities were directed to conducting a NFκB dose response and time course experiment using Apo-2-ligand (Apo-2L) and TNF.

128. I conducted the experiments using HeLa cells which I set up on March 17, 1997 (I made an error in writing that I set up the cells on “3/18/96”).

129. In part, the purpose of these experiments was to determine whether Apo-2L alone can regulate NFκB activity and to determine if the NFκB activation was dose-dependent, time-dependent, or both. NFκB activation is implicated in inflammatory responses as well as apoptosis. NFκB exists in the cellular cytoplasm in an inhibited state. Once stimulated, NFκB is released from its inhibited state, migrates to the nucleus of the cell, and activates transcription of a variety of genes. This activation can be measured using the techniques I used in my NFκB experiments. An increase in the amount of NFκB message in extracts reflects that it has been activated. Therefore, the purpose of my NFκB experiments was to evaluate the role of the Apo-2 receptor in activating NFκB activity.

130. My activities are reflected in the entries I made on page 71, an image of which is set forth below. My entries reflect that in wells 1-6, I incubated HeLa cells with 1ug/ml of Apo-

2L for different periods of time (i.e., 0, 10, 20, 30, 1 hour and 2 hours).

NFkB		Dose response		Project No. _____
TITLE		Time Course		Book No. 26865
From Page No. _____		To Page No. _____		71
3/17/97		Used HeLa cells		set up on 3/18/96
well #	Ligand	Conc.	Time	
1	Apo2L	ng/ml	0	
2	"	"	10 min	
3	"	"	20 "	
4	"	"	30 min	
5	"	"	1 hr	
6	"	"	2 hr	
TIME		1:10		

131. My entries reflect that in wells 13-18, I added serial dilutions of Apo-2L to the wells and incubated the cells and ligand for 30 minutes as reflected in the entries below. The Apo-2L concentrations ranged from 0 ng/ml to 1000 ng/ml.

13	Apo2L	0	30 min
14	"	0.1 ng/ml	"
15	"	1 "	"
16	"	10 "	"
17	"	100 "	"
18	"	1000 "	"

132. Following the incubations, I set up the NFkB assay per my "usual" protocol and loaded the gels as I described in the notebook page under the "Gel loading" entry. These activities are reflected in the image of my notebook entries below. I further noted "73" under the "To Page No\_\_" entry reflecting that I recorded additional data relating to this experiment on page 73.

SET UP NFkB assay as usual									
Gel loading	1	2	3	4	5	6	7	8	9
	10	11	12	13	14	15	16	17	18
	19	20	21	22	23	24			
									To Page No. 73

## 12. Page 73, Notebook 26865 (ADE-24)

133. Page 73 does not contain any entries on the page. However, I taped a pocket on page 73 and inserted five autoradiographs and/or images of gels from the experiments I

described on page 71.

134. I wrote March 20, 1997, on two of the autoradiographs, reflecting that I read the data on at least those autoradiographs on March 20, 1997. The data contained on the autoradiographs reflect that TNF induced a faster NFκB response (within first ten minutes) when compared to Apo-2L (maximum response at approximately thirty minutes).

**13. Pages 72 and 76 of Notebook 26865 (ADE-24)**

135. I conducted and recorded the activities described on pages 72 and 76 on March 20 and 21, 1997, as reflected by the dates recorded on that page.

136. My entries on page 72 reflect that I set up HeLa cells for use in NFκB experiments and transfected the cells with DNA encoding the caspase inhibitors “DEVD”, “ZVAD”, or “CRMA” or a “TRAF2” dominant negative mutant (which is an adaptor protein involved in the apoptosis signaling) to evaluate the effects of these proteins on NFκB activation in the presence of ligands including Apo-2L. I recorded these activities on page 72 of my notebook.

137. My entries on page 72 reflect that I treated wells numbered 2, 5, 8, 11, 14 and 17 with Apo-2L. I pretreated cells in well 2 with PBS alone (as a control), cells in well 5 with DVED, cells in well 8 with ZVAD, and I had transfected cells in well 11 with the pRK5 control vector, cells in well 14 with CRMA, and cells in well 17 with the TRAF2 dominant negative mutant.

138. I then conducted an NFκB assay on the cells I prepared.

139. On March 21, 1997, as described on page 76, I conducted additional NFκB experiments using HeLa cells.

140. The purpose of this additional experiment was to evaluate whether Apo-2 ligand induced expression of protective NFκB genes.



141. The image from page 76 below reflects that I used HeLa cells that I prepared on March 20, 1997, which I had plated in six-well culture dishes. I noted that certain cells were pretreated with either 1) PBS (phosphate buffered saline); 2) ALLN (an NFκB inhibitor); or 3) cyclohexamide (a transcription inhibitor), prior to addition of either PBS, Apo-2L, or TNF.

Project No. \_\_\_\_\_  
Book No. 26865 TITLE \_\_\_\_\_

76

From Page No. \_\_\_\_\_

3/21/97 Used HeLa cells set up 3/20/97 6well Dishes.

	Pre-Treat	Ligand conc + time
1	PBS	PBS
2	"	Apo2L 1ng/ml 30min
3	"	TNF 1ng/ml "
4	ALLN 100ng/ml 2hr	PBS
5	"	Apo2L 1ng/ml 30min
6	"	TNF 1ng/ml "
7	Cyclohexamide 50ng/ml 2hr	PBS
8	"	Apo2L 1ng/ml 30min
9	"	TNF 1ng/ml "

400ng/ml  
1000x stuck  
1000x stuck

142. I listed the pretreatment regimen on the left side of page 76 under the "pretreat" entry. I listed the ligands I added to the cells to the right of the pretreat column under the "Ligand" and "conc + time" entries. The latter entry reflects the concentration of ligand added and the duration of the incubation period.

143. I made the entry below at the bottom of page 76 reflecting that the results from the assay relating to the ALLN and cyclohexamide pretreatment experiments are included on page 77 of my notebook 26865.

For NFκB assay of 1-27 go to pg # 77			
For SARK assay of 10-27			
Witnessed & Understood by me,	Date	Invented by	Date

To Page No. \_\_\_\_\_

#### 14. Page 77, Notebook 26865 (ADE-24)

144. On March 21, 1997, I noted on page 77 of my notebook 26865 that "NFκB Assay

Set up as usual” reflecting that I set up the NFκB assay per my usual protocol. This is the only entry I made on page 77.

145. I taped a pocket at the bottom of page 77 and included images of the gels obtained from the NFκB assay in the pocket. The results from the gels were inconclusive.

Project No. 26865		77
Book No. 26865		
TITLE		
From Page No.	NFκB Assay	
3/21/97	Set up as usual	

15. Page 87, Notebook 26865 (ADE-24)

146. I conducted and recorded the activities described on page 87 on March 28, 1997, as reflected by the dates recorded on that page.

147. My records reflect that my activities were directed to transfecting 293 cells with DD2-IgG fusion proteins and detecting whether the fusions were expressed by the cells.

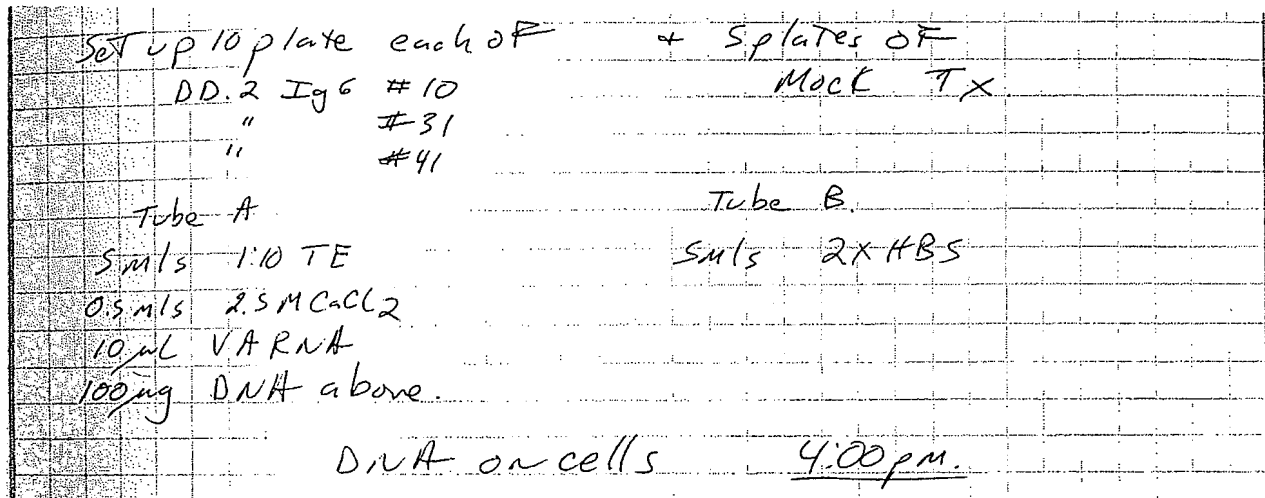
148. I made the entries set forth in the image below across the top of page 87 reflecting that I conducted a transfection experiment using DD.2-IgG DNA constructs (“Tx of DD.2-IgG”) and that I “used 35 100mm Dishes of 293s cells” in my experiments.

Tx of DD.2-IgG.		Project No. 26865	87
Book No. 26865			
TITLE			
From Page No.	used 35 100mm Dishes of 293s cells. Fed cells 11:00 a.m.		
3/28/97			

149. The DD.2-IgG DNA I used in these experiments was first described on pages 4-5 of Maya Skubatch’s notebook 27236. Ms. Skubatch conducted these experiments under my direction and supervision, and I describe her activities in a later section of this declaration.

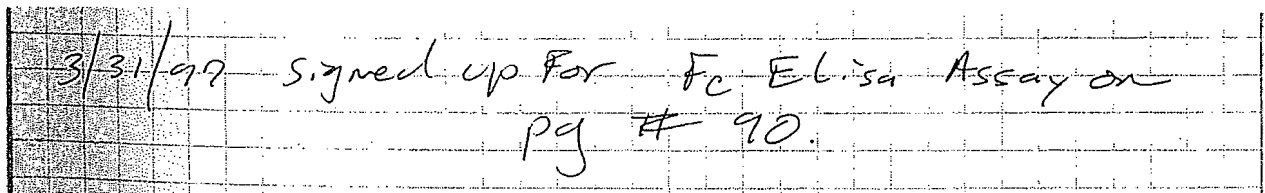
150. The entries I made on page 87 of my notebook reflects that I set up ten plates of three DD.2-IgG constructs (#10, #31, and #41) as well as five “plates of Mock Tx,” which

reflects that only the prk5 vector was used in the mock transfections. I also recorded the reagents used for the transfections as reflected below the "Tube A" and "Tube B" entries, and that I transfected the 293 cells with the various DD2-IgG fusion constructs and control DNA constructs. The image of page 87 set forth below reflects these activities.



151. On March 29, 1997, I noted that I "fed cells serum free media," reflecting that I replaced the media with serum free media to the 293 cells that I transfected with the DD.2-IgG construct.

152. On March 31, 1997, I made the entry in the image below which reflects that I "signed up for Fc ELISA Assay" and recorded more information about the ELISA request on page 90 of my notebook 26865. These entries reflect that I requested the Genentech Assay Group to conduct an ELISA of the supernatant from the cells I transformed to determine whether the "Fc" region of the DD2-IgG fusion protein could be detected by ELISA analysis.



**16. Page 90, Notebook 26865 (ADE-24)**

153. I did not record any activities on page 90 of my notebook 26865. However, I

taped the ELISA request form, that I described on page 87, onto page 90.

154. The ELISA request form includes the following information as reflected in the image of page 90 below: 1) that I submitted the samples (reflected by the notation at arrow “A”); 2) that the “Assay” was an “Fc Elisa” (reflected by the notation at arrow “B”); 3) that the date submitted and date to be assayed was “3/31/97”; and 4) that the product ID was “DD.2-IgG” (reflected by the notation at arrow “D”).

A
B
C
D

10

Project No. \_\_\_\_\_  
Book No. 26865 TITLE \_\_\_\_\_

From Page No. \_\_\_\_\_

**ASSAY REQUEST/REPORT FORM**  
**Genentech, Inc.**

ASSAY: Fc Elisa

To sign up for assays call x2632.  
Drop off samples in room 5295.

GREY TO BE COMPLETED BY ASSAY SERVICES							
A.S. EXPT. NO.				DATE			
A.S. TECH.				TEST PROCEDURE			

SAMPLES SUBMITTED BY <u>Scott Morris</u>		EXTENSION <u>18-119</u>	MAILSTOP # <u>40</u>	COST CENTER <u>4124</u>	PROJECT CODE <u>1110</u>	PRODUCT I.D. <u>DD.2-IgG</u>	# SAMPLES <u>2</u>
DATE SUBMITTED <u>3/31/97</u>	DATE TO BE ASSAYED <u>3/31/97</u>	<input type="checkbox"/> WILL PICK UP <input type="checkbox"/> MAIL RESULTS		<input type="checkbox"/> E-MAIL RESULTS (Log in: _____) <input type="checkbox"/> FAX RESULTS (# _____)		<input checked="" type="checkbox"/> CALL ME STORAGE TEMP. (PRE-ASSAY): <input type="checkbox"/> AMBIENT <input type="checkbox"/> -10°C OR BELOW <input checked="" type="checkbox"/> 2° - 8°C <input type="checkbox"/> OTHER	
<input type="checkbox"/> GMP <input type="checkbox"/> GLP Study No. _____ <input type="checkbox"/> N/A	SAMPLE MATRIX <u>PBS</u>	ANTICOAGULANT	<input type="checkbox"/> RADIOACTIVE <input type="checkbox"/> TOXIC HAZARD <input type="checkbox"/> BIOHAZARD (NON-HUMAN/NON-PRIMATE) Specify type and amount: _____		SAMPLE OF HUMAN/PRIMATE ORIGIN? <input type="checkbox"/> NO <input checked="" type="checkbox"/> YES If yes, identify source: <u>298 cells</u> <small>(specify tissue, cell line, blood, etc.)</small>		
SAVE SAMPLES? <input type="checkbox"/> YES: Storage Temp. _____ <input checked="" type="checkbox"/> NO: Samples not picked up within 2 weeks will be discarded.		NOTES: _____					

155. I listed the samples and the dilution of each sample to be assayed on the bottom portion of the assay request form. For example, sample numbers 1-4 are designated #10, which reflects that the samples contained supernatant from cells transfected with the DD2-IgG construct designated “#10.” The entries under the “Dilution” column for samples 1-4 read “neat, 1:10, 1:100, and 1:1000”, respectively, reflecting that sample 1 was not diluted, sample 2 was a 1:10 dilution of sample and buffer, sample 3 was a 1:100 dilution of sample and buffer, and sample 4 was a 1:1000 dilution of sample and buffer.

156. I taped the raw ELISA data obtained from the Assay group on the lower right side of page 90. The results reflect that samples 17 and 20 (neat #41) gave a weak positive signal in the ELISA.

**17. Page 91, Notebook 26865 (ADE-24)**

157. I conducted and recorded the activities described on page 91 on March 31, 1997, as reflected by the dates recorded on that page.

158. My records reflect that my activities were directed to transfecting 293 cells with DD2 DNA and co-transfecting the cells with constructs containing either DVED (a caspase inhibitor), ZVAD (a caspase inhibitor), CRMA (a caspase inhibitor) or hFADD D/N (a human dominant negative adaptor protein).

159. The purpose of co-transfecting the cells with the caspase inhibitors was to evaluate whether the induction of NF $\kappa$ B by expression of the DD2 DNA was mediated through caspase activity in the 293 cells. If there was no observed increase following co-transfection with DD2 DNA and the caspase inhibitor constructs, it would be reasonable to conclude that the protein encoded by the DD2 DNA induced NF $\kappa$ B through a pathway involving caspases.

160. The purpose of co-transfecting the cells with human FADD D/N (dominant negative) was to evaluate whether DD2 – mediated induction of NF $\kappa$ B involved the FADD adaptor protein. The dominant negative form of FADD used in these experiments was known to block apoptosis induction by overexpression of other TNF family receptors in the cells. If expression of DD2 induced NF $\kappa$ B when co-transfected with FADD D/N, that would reflect that the protein encoded by DD2 DNA signaled independently of the FADD adaptor protein.

161. I made the entry below across the top of page 91 reflecting that I set up the transfection experiments with the 293 cells.

Project No. _____		Book No. <u>26865</u>	91
TITLE _____			
From Page No.	3/31/97 SET up Tx of 293S cells.		
	60mm Dishes. Fed cells 11:00am.		

162. The entries I made on page 91, set forth in the image below, provide the plate numbers containing the DNA I used to transfect the cells. To the right are brackets reflecting the caspase inhibitors that I co-transfected into the cells on to the plates (4-6 – DVED; 7-9—ZVAD; and 10-12 –CRMA). Plates 13-15 were co-transfected with DNA encoding a dominant negative mutant of the human FADD adaptor protein. Plates 1-3 were not co-transfected with additional constructs.

plate #		
1	PrKS	
2	DD.2	
3	TNPR1	
4	PrKS	+ DVED 200µM
5	DD.2	
6	TNPR1	
7	PrKS	+ ZVAD 200µM
8	DD.2	
9	TNPR1	
10	PrKS	+ CRMA
11	DD.2	
12	TNPR1	
13	PrKS	+ h FADD D/N
14	DD.2	
15	TNPR1	

163. I recorded the reagents I used to conduct the transfection experiment and noted at the bottom of page 91 that “DNA on cells,” reflecting that I transfected the cells with the various DNA constructs described on page 91. My entries are reflected in the image of page 91 below. I noted that I recorded additional information related to these experiments on page 92 (“To Page

No. 92).

Tube A	Tube B
0.5 mL 1:10 T.E.	0.5 mL 2xHBS
50 µL 2.5 M CaCl <sub>2</sub>	
1 µL VPRN	
10 µg DNA above	
10 µg CRMA or hFADD D/N if necessary	
Add 300 µL / plate	
DNA on cells 4:00 p.m.	

To Page No. 92

18. Pages 92-93, Notebook 26865 (ADE-24)

164. I conducted and recorded the activities described on pages 92 and 93 on April 1, 1997, as reflected by the dates recorded on those pages.

165. My records reflect that my activities were directed to conducting an NFκB assay using cells that I previously co-transfected with DD2 DNA and constructs encoding either DVED (a caspase inhibitor), ZVAD (a caspase inhibitor), CRMA (a caspase inhibitor) or hFADD D/N (an adaptor protein) as described on page 91.

166. The entries I made on page 92 reflect that I set up samples for the NFκB assay per my usual protocol, as reflected in the image below.

Project No. \_\_\_\_\_  
Book No. 26865 TITLE \_\_\_\_\_

92

From Page No. 91

4/1/97 worked up samples for NFκB Assay As usual

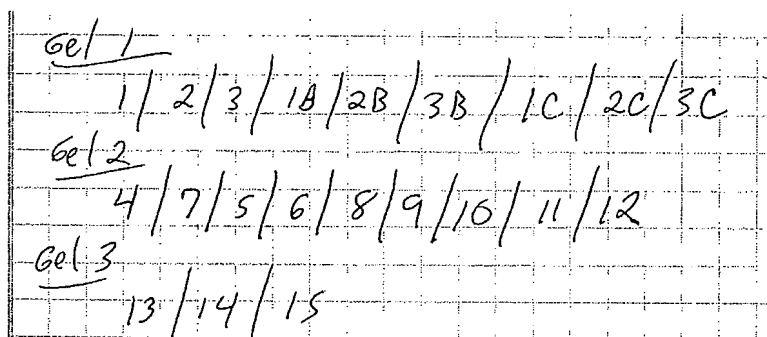
167. On the left side of the page I listed the cell samples I used, which correspond to the samples I described on page 91. I noted that I added the NFκB probe to all the samples and that I added "2ul Cold" to samples 1B-3B and 2ul of "α-NFκB" antibody to samples 1C-3C. I added the "cold" NFκB as a control to compete with the radioactive NFκB probe to ensure that a

positive result was a bona fide positive and not due to non-specific probe binding. I added the NFκB-specific antibody to “supershift” NFκB protein in the sample which serves as a positive control. The antibody-NFκB complex would be a larger complex and migrate slower than NFκB alone on a gel. Seeing a “supershifted” NFκB-antibody band at the top part of a gel, and a non-shifted NFκB at a farther migration point on the gel, would provide confirmation that the results from the assay were reliable.

Samples			NFκB Probe	
1	PKS		"	
2	DD.2		"	
3	TNFR1		"	
4B	PKS	From #1	"	+ 2ul Coled
5B	DD.2	From #2	"	"
6B	TNFR1	From #3	"	"
1C	PKS	From #1	"	+ 2ul αNFκB AB
2C	DD.2	From #2	"	"
3C	TNFR1	From #3	"	"
4	PKS	} + DVED	"	
5	DD.2		"	
6	TNFR1		"	
7	PKS	} + ZVAD	"	
8	DD.2		"	
9	TNFR1		"	
10	PKS	} + CRMA	"	
11	DD.2		"	
12	TNFR1		"	
13	PKS	} + h Padd	"	
14	DD.2		"	
15	TNFR1		"	

168. I loaded the gels with the samples according to the order described at the bottom of page 92.





169. I was unable to locate the images of the gels I ran from this series of experiments in my notebooks. Therefore, I am unable to comment on the results I obtained.

**19. Page 43, Notebook 26119 (ADE-25)**

170. From April 7-9, 1997, I attended a scientific conference and did not conduct any laboratory work during this time.

171. On April 10, 1997 I noted "Back From Asilomar" on page 43. This notation reflects that I returned to work in the laboratory after attending the conference.

172. I conducted and recorded the activities described on page 43 on April 10, 1997, as reflected by the dates recorded on that page.

173. My records reflect that my activities were directed to constructing a DD.2 extracellular domain (ECD) construct and making an Apo-2-IgG construct and expressing the constructs in cells.

174. As reflected in the image below, I noted that before we left for the scientific conference, "Maya set up PCRs of DD.2 ECD with SM.1 or SM.2 + SM.4." These entries reflect that Maya Skubatch set up PCR experiments using the extracellular domain sequence of the DD.2 DNA and PCR primers designated SM.1, SM.2, SM.4. I designed these primers as reflected by my initials ("SM") used in the primer designation and the sequence of the primers are submitted in **ADE-31** and **ADE-32**. The SM.1 and SM.2 primers corresponded to sequence on the 5' end of the "met" start site (SM.1) and overlapping with the "met" start site (SM.2), at

the 5' end of the extracellular domain. The SM.4 primer corresponded to sequence close to the predicted transmembrane domain of the DD.2 sequence at the 3' end of the extracellular domain. The purpose of the experiment was to amplify DD.2 extracellular domain sequences using PCR. I listed the tubes containing the primers and the DD.2 DNA. I noted that I used either 1:10 or 1:100 dilutions of DD.2 DNA in these experiments.

Project No. \_\_\_\_\_  
Book No. 26119 43

TITLE \_\_\_\_\_


From Page No. \_\_\_\_\_ Back From Asilomar

4/10/97 Before we left Maya set up PCRs of DD.2 ECD with SM.1 or SM.2 + SM.4

	SM.1	SM.4	DD.2	Diluted	
1	SM.1	SM.4	DD.2	Diluted	1:10
2	SM.2	SM.4	"	"	"
3	" .1	SM.4	"	"	1:100
4	" .2	SM.4	"	"	"

175. My continued activities are reflected in the image below. I noted that I "took PCRs above + Ran a Gel." I taped a photograph of the gel I ran on the left side of the page and described the experimental conditions under which I ran the gel on the right side of the page.

I Took PCRs above + Ran a Gel



I digested  
#3 + #4 with  
90  $\mu$ L PCR  
10  $\mu$ L 10X RB 100 mM  
2  $\mu$ L BSTE II  
20 min  
55° 3 h  
+ 2  $\mu$ L RI  
+ 2  $\mu$ L PVU I  
37° O.N. Jane put  
in Freezer.

Also I ordered primers to make DD.2 ECD-Flag Tag  
will be ready tomorrow after noon

176. As reflected toward the bottom of page 43, I noted that “Also I ordered primers to make DD.2-ECD-FlagTag will be ready tomorrow afternoon.” These entries reflect that I ordered primers for use in making a DD2.-ECD-FLAG fusion protein wherein the extracellular domain of the DD.2 molecule would be ligated to a FLAG tag. A FLAG tagged protein is useful for conducting immunoprecipitation and binding assays.

**20. Page 44, Notebook 26119 (ADE-25)**

177. I conducted and recorded the activities described on page 44 on April 13, 1997, as reflected by the dates recorded on that page.

178. My records reflect that my activities were directed to constructing a DD.2-ECD-FLAG construct.

179. Across the top of page 44, I wrote “PCRs of DD.2 ECD.Flag” reflecting that the experiments I conducted were PCR experiments designed to make a DD.2-ECD-FLAG fusion construct.

Project No. _____			
44	Book No. 26119	TITLE _____	
From Page No. _____			
4/13/97	PCRs. of	DD.2 ECD.Flag	

180. The DD.2 DNA used in these experiments was obtained from the PCR experiments I described on page 43.

181. Directly below the entry in the image above, I listed the reagents I used in the PCR reaction. The 5' primer was either “SM.1” or “SM.2” and the 3' primer was a DD.2 Flag primer. My entries are reflected in the image below.

1 $\mu$ l	DNA		
10 $\mu$ l	10X PCR Buffer		
24 $\mu$ l	1.25 mM dNTPs		
1 $\mu$ l	5' primer	SM.1 or SM.2	
1 $\mu$ l	3' primer	DD.2 Flag ECD	
63 $\mu$ l	ddH <sub>2</sub> O		
1 $\mu$ l	Tag		

Tube #			DNA
1	SM.1	DD.2 Flag ECD	DD.2 neat
2	SM.2	"	" "
3	SM.1	"	" 1:10
4	SM.2	"	" "

↑  
A

182. The image above reflects that I recorded the contents of the reaction tubes numbered 1 through 4 (reflected by the notation at arrow "A") which included either a 5' primer (SM.1 or SM.2), a 3' primer (DD.2 Flag), and DD.2 DNA either "neat" (non-diluted) or diluted 1:10. (Diluting the samples may reduce the error rate when conducting the PCR experiment).

183. I ran the PCR experiment, Dr. Sheridan added additional reagents to digest the reaction that afternoon, and the reaction incubated at 37 degrees Celsius overnight. These activities are reflected on page 44.

## 21. Page 94-96 Notebook 26865 (ADE-24)

184. I conducted and recorded the activities described on pages 94-96 on April 14 and 15, 1997, as reflected by the dates recorded on those pages.

185. My records reflect that my activities were directed to conducting NF $\kappa$ B experiments using a DD2.1 construct.

186. On April 14, 1997, I made a radioactive NF $\kappa$ B probe for use in the NF $\kappa$ B assays I conducted. I recorded these activities on page 94 of my notebook and included the reagents I used to make the probe and the printout from the scintillation counter measuring the radioactivity (cpm) of the probe that I made.

187. The probe had approximately  $1.7 \times 10^6$  cpm of radioactivity which was sufficient for me to use in the NF $\kappa$ B assay.

188. On April 14, 1997, I also transfected 293S cells with DNA encoding DD2 and prepared samples for analysis by NF $\kappa$ B assay as reflected by the entries I made on page 95 of my notebook, set forth in the image below.

293S TX		Project No. _____	Book No. <u>26865</u>	95
TITLE _____				
From Page No. _____	4/14/97 Set up TX 100 mM Dishes. Fed cells 9:00 am.			

Plate #		Tube A	Tube B
1	Prk5	0.5 ml/s 1:10 TE	0.5 ml/s 2xHBS
2	DD.2	50 $\mu$ l 2.5M CaCl <sub>2</sub>	
3	TNFR1	10 $\mu$ g DNA	
4	Prk5	1 $\mu$ g VARNA.	
5	DD.2		
6	TNFR1		
7	Prk5		
8	DD.2		
9	TNFR1		

+ ZVAD (plates 4-6)

+ Prk CRMA (plates 7-9)

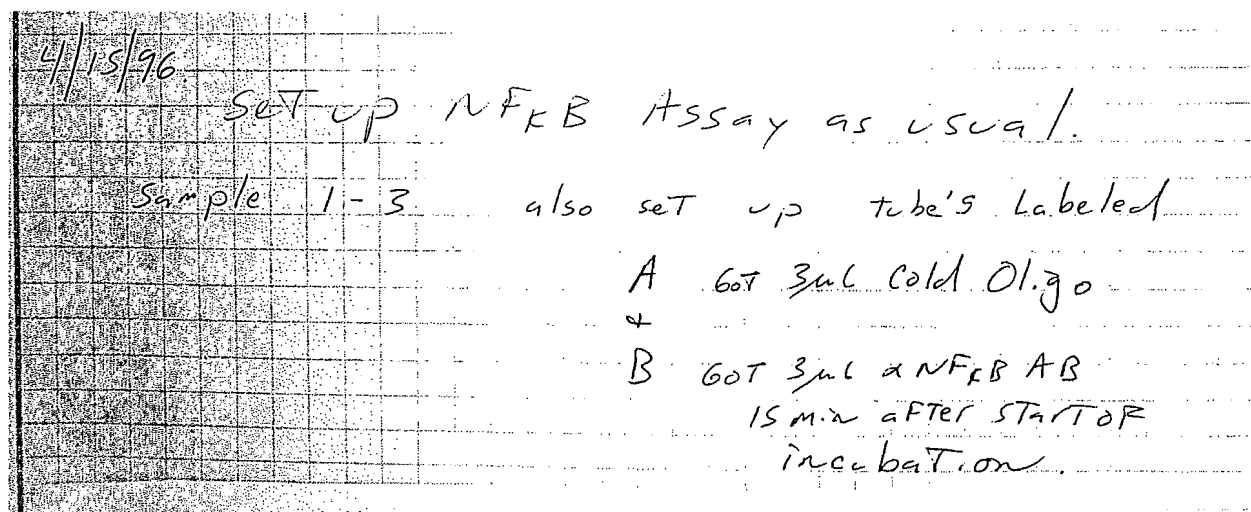
DNA on cells 12:00 noon.

Jamie Fed cells Fresh media at 9:00 pm + put ZVAD on plates 4-6

189. My entries reflect that I recorded the plate number (1-9) and DNA I used to transfect the cells (prk5, DD.2 or TNFR1) on the left side of the page. I noted that the caspase inhibitor ZVAD was added to plates 4-6 and that prk-CRMA was co-transfected with the other DNA on plates 7-9. I recorded the reagents I used on the right side of the image above in tubes A and B and further noted that "Jamie Fed cells Fresh media at 9:00pm + put ZVAD on plates 4-6." These entries reflect that Dr. Sheridan ("Jamie") fed the cells and was the person who added the ZVAD caspase inhibitor to plates 4-6.

190. My entries reflected in the image below correspond to activities I conducted and

recorded on April 15, 1997, and not 1996. The entries I made on page 95 reflect that I set up another NF $\kappa$ B assay and that I used tubes having cold (non-radioactive) probe in a set of tubes labeled A, and tubes having an NF $\kappa$ B-specific antibody in a set of tubes labeled B. I noted that the NF $\kappa$ B antibody should be added "15 min after start of incubation." The purpose of adding the cold oligonucleotide was to ensure that any NF $\kappa$ B probe hybridization observed was specific. As described above, the NF $\kappa$ B antibody was added to evaluate a shift in mobility of the NF $\kappa$ B band and served as a positive control in the experiment.



4/15/96  
Set up NF $\kappa$ B Assay as usual.  
Sample 1-3 also set up tube's labeled  
A GOT 3 $\mu$ L cold Oligo  
+  
B GOT 3 $\mu$ L  $\alpha$  NF $\kappa$ B AB  
15 min after start of  
incubation.

191. The results from these experiments are reflected in images of gels inserted in a pocket on page 95. The gels suggested that there may have been some NF $\kappa$ B activity, although the results were generally not conclusive.

192. On April 14, 1997, I also conducted HeLa cell transfections and set up NF $\kappa$ B experiments in parallel to the experiments described above using 293 cells.

193. As reflected in the image below, I recorded the plate number (1-9) and DNA I used to transfect the cells (prk5, DD.2 or TNFR1) on the left side of page 96. I noted that the caspase inhibitor ZVAD was co-transfected in the cells in plates 4-6 and that prk-CRMA was co-

transfected with DNA used to treat the cells on plates 7-9. On the right side of the page I noted that I put the DNA on cells, fed them with fresh media, and added ZVAD to tubes 4-6.

Plate #		
1	PrKS	
2	DD.2	
3	TNFR1	
4	PrKS	+ ZVAD
5	DD.2	
6	TNFR1	
7	PrKS	+ PrK CRMA
8	DD.2	
9	TNFR1	

194. The entry I made toward the middle of page 96, provided in the image below, reflects that I set up another NF $\kappa$ B assay on April 15, 1997, and that I used tubes having cold (non-radioactive) probe in a set of tubes labeled A and tubes having an NF $\kappa$ B-specific antibody in a set of tubes labeled B. I noted that the NF $\kappa$ B antibody should be added "15 min after start of incubation." The purpose of adding the cold oligonucleotide was to ensure that NF $\kappa$ B probe hybridization observed was specific, and the purpose of adding the antibody was to evaluate a shift in mobility of the NF $\kappa$ B, as described previously.

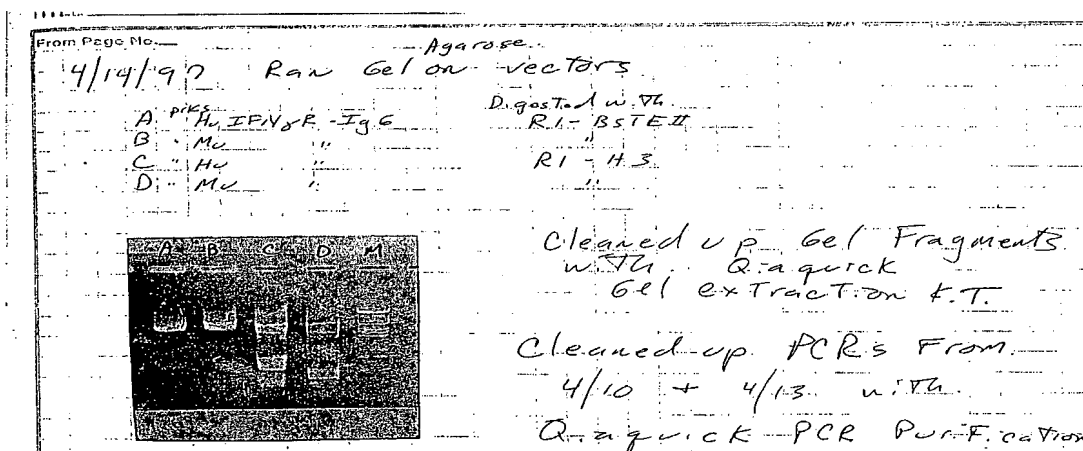
4/15/97 set up NF $\kappa$ B as usual.  
samples 1-3 also set up as  
A GOT 3 $\mu$ l cold oligo  
+  
B GOT 3 $\mu$ l  $\alpha$  NF $\kappa$ B AB  
15 min after start of  
Incubation.

195. The results from these experiments are reflected in images of gels inserted in a pocket on page 96. The gels reflect that when cells were transfected with DD.2, NFκB increased, and that if they were also treated with the caspase inhibitor ZVAD, the amount of NFκB further increased. This appeared to suggest that the NFκB activity increased when apoptosis was blocked by the caspase inhibitor ZVAD, but not by CRMA.

**22. Page 45, Notebook 26119 (ADE-25)**

196. I conducted and recorded the activities described on page 45 on April 15, 1997, as reflected by the dates recorded on that page.

197. My records reflect that I continued my work on PCR experiments from April 10 and April 13, 1997, directed to making DD.2 fusion constructs (DD.2-ECD Flag and DD.2-IgG constructs). I noted that I “cleaned up PCR’s from 4/10 + 4/13 with Qiaquick PCR purification” as reflected in the image below.



198. The entries I made at the top of page 45 reflect that I digested vectors containing the human and murine interferon alpha-IgG fusion DNA using the restriction enzymes EcoR1 (“R1”) and BSTEII (samples A and B), or using the restriction enzymes EcoR1 and HINDIII (“H3”).

199. After digestion of the vectors, I ran the digested vector DNA on a gel and



“cleaned up gel fragments with Qiaquick gel extraction kit” as reflected by my entries on page 45.

200. Once I “cleaned up” both the gel fragments containing the vector, and the PCR products from the experiments I conducted on April 10 and 13, I set up “ligations” to ligate either 1) DD.2 with IgG sequence in the vectors I digested at the top of page 45; or 2) DD.2-ECD with FLAG sequences into plasmid vectors. Following the ligations, I transformed JM109 bacterial cells with the ligated vectors. These activities are reflected in the image of my notebook page below.

Set up ligations Quick				2ul Tube 2 1ul vector 2ul Frag Mix	19ul Tube 1 Mix 1ul Tube 3 5ul RT + Transform
P.C.K.S.	1u IFN $\gamma$ R-IgG	R1-H3	+	1-4 From 4/13	
"	MU			1-4	"
P.C.K.S.	1u IFN $\gamma$ R-IgG	R1-BsTET	+	3+4 From 4/10	
Transformed into JM109s					
4/15/97	picked 1-10 colonies		DD.2-IgG		
	11-82		DD.2-Flag-ECD		
To Page No. 46					
Witnessed & Understood by me,		Date	Invented by		Date
			Recorded by		

201. The entries at the bottom of page 45 next to “4/15/97” reflect that on April 15, 1997, I picked bacterial colonies that I believed to be expressing the DD.2.1-IgG or DD.2.-Flag-ECD DNA. I designated colonies possibly containing DD.2-IgG DNA as “1-10,” and colonies possibly containing DD.2-Flag-ECD DNA as “11-82.” I further noted that I recorded additional information relating to these experiments on page 46 as indicated by the “To Page No. 46” entry.

### 23. Pages 46-48, Notebook 26119 (ADE-25)

202. I conducted and recorded the activities described on pages 46-48 on April 15 and

16, 1997, as reflected by the dates recorded on those pages.

203. My records reflect that my activities were directed to making DD.2 IgG or Flag fusion constructs.

204. I noted at the top of page 46 that "Setup PCRs of DD.2 IgG or Flag-ECD." The DD.2-IgG entry has a "1" in a circle above it and the Flag-ECD entry has a "2" in a circle above it. These numbers reflect the protocols that I followed for the IgG fusion work (1) or the Flag-ECD fusion work (2) as reflected on page 46 and in the image below.

Project No. \_\_\_\_\_  
Book No. 26119 TITLE \_\_\_\_\_

46

From Page No. \_\_\_\_\_

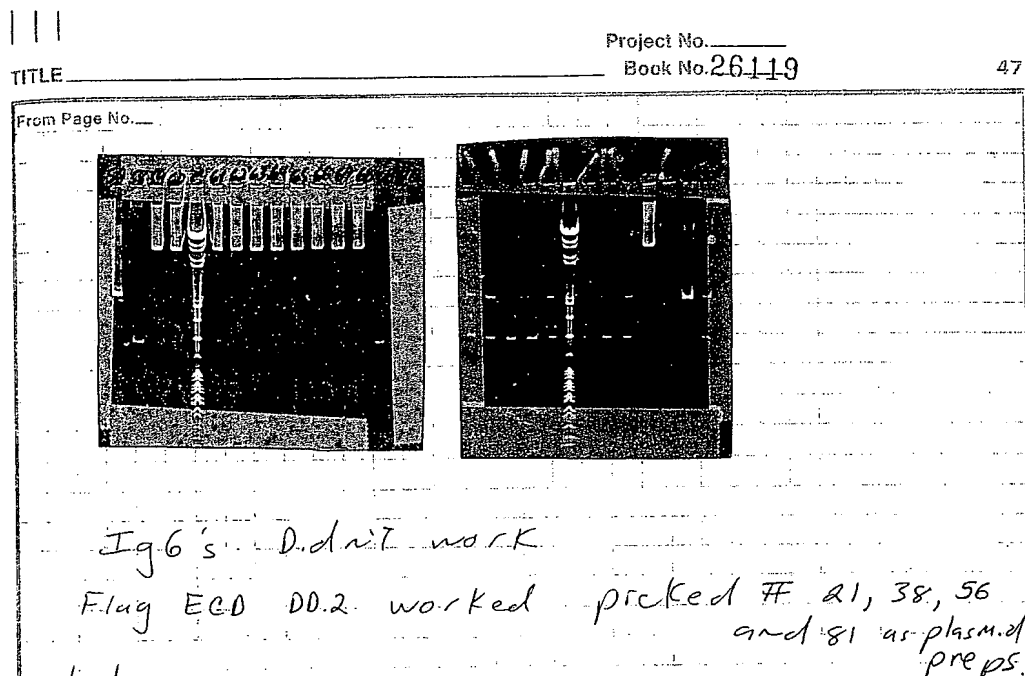
①		②	
4/15/97 Setup PCRs of DD.2 IgG or Flag-ECD			
	X12		X80
20µL dH <sub>2</sub> O + Colony	—	20µL dH <sub>2</sub> O + Colony	—
5µL PCR Buffer	60	5µL PCR Buffer	400
12µL 1.25 mM dNTPs	144	12µL 1.25 mM dNTPs	960
0.5µL U.IgG P4	6	0.5µL P4	40
0.5µL P4 R	6	0.5µL P4 R	40
12µL dH <sub>2</sub> O	144	12µL dH <sub>2</sub> O	960
0.5µL Taq	6	0.5µL Taq	40

205. The purpose of these experiments was to use PCR to amplify the DD2-IgG sequences and DD2-ECD-Flag DNA sequences to determine if the plasmids contained inserts. The "X12" and "X80" entries reflect the number of tubes I used for the PCR reactions and the entries below the "X12" and "X80" entries reflect the volume of each reagent I needed for the number of reaction tubes I used in the experiment.

206. Following the PCR reactions, I ran the PCR reaction products on gels and attached photographs of the gels to pages 46 and 47 of my notebook. I labeled each lane on the gels identifying the DNA picked from positive colonies 1-82 listed at the bottom of page 45.

207. I noted at the middle of page 47 that "IgG's Didn't work" indicating that the PCR amplification of the DD2-IgG fusion described on page 46 was unsuccessful. I noted that "Flag-

ECD DD.2 worked picked #21, 38, 56, and 81 as plasmid preps.” These entries reflect that the PCR amplification of the Flag-ECD DD.2 was successful and that I picked DNA corresponding to colonies 21, 38, 56, and 81 for use in making plasmid preparations of the DNA from those clones. These entries are reflected in the image of page 47 below.



208. On April 16, 1997, I noted at the middle of page 47 that I “worked up plasmid preps.” This entry relates to the plasmid preparations referenced immediately above on page 47 and reflects that I made plasmid preparations for the DNA in lanes 21, 38, 56, and 81. An image of these entries is provided below.

P.C.P.S.

4/16/97 worked up Plasmid preps.

Set up Digests of Plasmids to make IgG-DD2 intermediate.  
Then Do Mutagenesis.

(1330 Bp)	(1320 Bp)	(1050 Bp)	(800 Bp)
3 $\mu$ L DD.2	3 $\mu$ L IL-1A IgG	3 $\mu$ L DD.2	3 $\mu$ L 2FC1
10 $\mu$ L 10x RB 75mM	10 $\mu$ L 10x RB 75mM	10 $\mu$ L 10x RB 75mM	10 $\mu$ L 10x RB 75mM
2 $\mu$ L RI	2 $\mu$ L XBA	2 $\mu$ L RI	2 $\mu$ L SacI
2 $\mu$ L XBA	2 $\mu$ L H3	2 $\mu$ L SacI	2 $\mu$ L HindIII
83 $\mu$ L dH <sub>2</sub> O	83 $\mu$ L dH <sub>2</sub> O	83 $\mu$ L dH <sub>2</sub> O	83 $\mu$ L dH <sub>2</sub> O
37° 2 hrs	37° 2 hrs	37° 2 hrs	37° 2 hrs

run 1% Agarose Gel

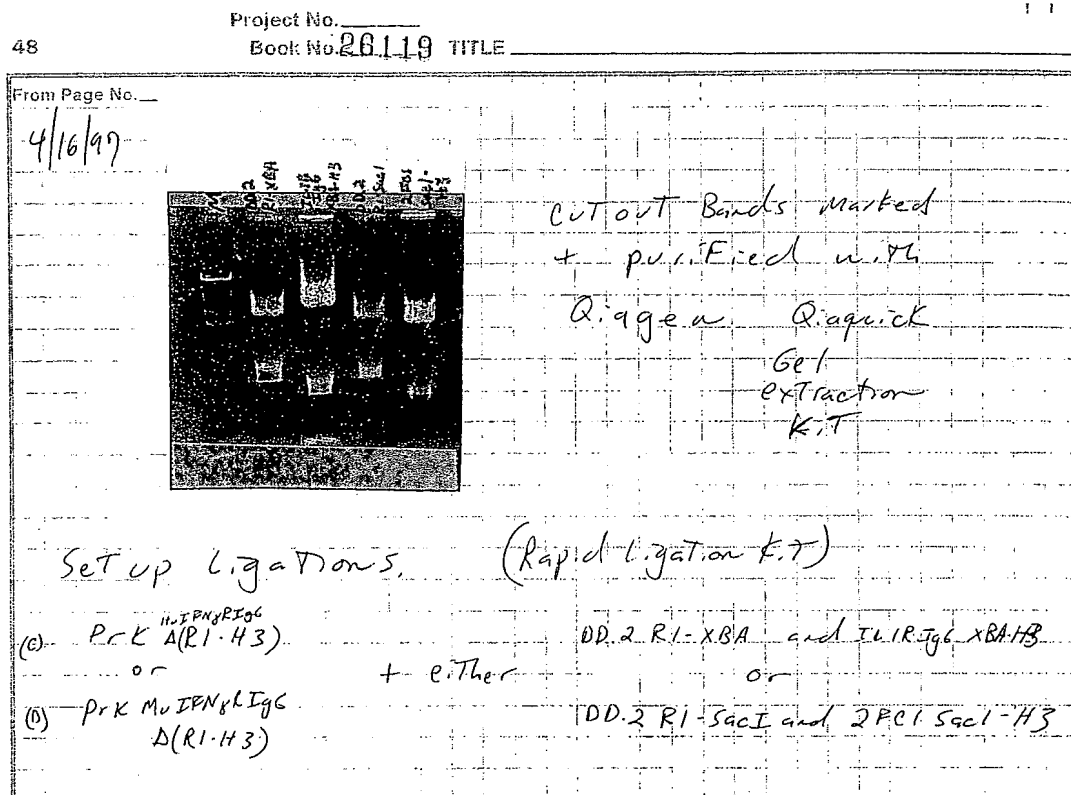
To Page No. \_\_\_\_\_

209. The notation at arrow "A" reflects that I further noted on page 46 "setup digests of plasmids to make IgG-DD2 intermediate then do mutagenesis." These entries reflect that I set up an experiment to digest plasmids that would be used to make a DNA construct encoding a DD2-IgG fusion intermediate. I planned to use the intermediate vector as an alternative approach to making a DD2-IgG fusion. The intermediate contained additional sequence that would ultimately be spliced out to bring the DD.2 and IgG portions of the DNA construct together. I proposed this approach because I was having difficulty obtaining a DD2-IgG fusion construct using traditional methods.

210. I ran the digested plasmids I prepared on a "1% Agarose Gel" as reflected by my entry at the bottom of page 47.

211. On page 48, my records reflect that I continued with the work described previously on pages 43-47. I noted at the top of page 48 that I "cut bands marked and purified with Qiagen Qiaquick Gel extraction kit." A photograph of the "marked" gel is taped at the top of page 48 and has four dots marking the bands to be excised from the gel. The marked bands are the four lowest bands on the image below. The bands I "cut out" and purified were the

plasmids I digested with various restriction enzymes as described on page 47. I noted at the middle of page 48 that I set up ligations which reflects that I ligated the restriction digested IgG donor sequences and DD.2 donor sequences (listed under “(Rapid Ligation Kit)”) into “host” plasmids I previously prepared, which I designated “(C) prkHuIFNRIgGΔ (R1-H3) and (D) prkMuIFNRIgGΔ (R1-H3).”



212. Following purifications of the DNA from the gel I ran, I set up ligations using a commercially available “Rapid Ligation Kit” to ligate DD.2 DNA, IgG DNA, and vector DNA to form intermediate vectors containing the DD2 and IgG inserts, and the additional inserts described on page 47 (“ILIRIgG XBA-H3” and “2Fc1 SacI-H3”). These activities are reflected in the image above.

213. After I completed the ligation experiment, I transformed JM109 *E. coli* cells with the newly ligated vectors.

214. Toward the bottom of page 48, I continued characterizing the DD.2-ECD Flag plasmids and made the following entries “4/16/97 Digest Plasmid Preps of DD.2 ECD Flag” reflecting that I digested DD.2-ECD Flag plasmid preparations that I made and described on page 47 of my notebook. The purpose of the digest was to confirm that the inserts in the plasmids were of the correct size. An image of notebook page 48 reflecting these activities is set forth below.

4/16/97 Digest Plasmid Preps of DD.2 ECD Flag			
1 $\mu$ L DNA	DNA's	21, 38, 56, 81	
2 $\mu$ L 10X RBE4MM	Enzymes	R1-H3	
1 $\mu$ L each enzyme	or	R1-SacII-H3	
15 $\mu$ L dH <sub>2</sub> O			
37 $^{\circ}$ O.N.			
4/16/97 new Digests (Backup)			
3 $\mu$ L DNA (DD.2 or PRK5 IL1R-IgG)			
10 $\mu$ L 10X RBE 25MM			
2 $\mu$ L each enzyme	R1 + XBA		
83 $\mu$ L dH <sub>2</sub> O			
37 $^{\circ}$ O.N.			
Witnessed by _____	Invented by _____	Date _____	To Page No. _____
	Recorded by _____		

215. My entries reflect that I used “DNAs 21, 38, 56, 81” which correspond with the description of DNA I made on page 47. I further noted under the “Enzymes” entry that I used “R1-H3” (EcoRI – HINDIII) “or R1-SacII-H3” (EcoRI-SacII-HINDIII) as the restriction enzymes used to digest the plasmids containing the DNA designated 21, 38, 56, and 81.

216. I made an additional entry toward the bottom of page 48 “new Digests (Backup)” which reflects that I conducted additional digests using vectors containing DD.2 DNA or “prk5 IL1R-IgG” DNA I described at the middle of page 48. I conducted these repeat digests to be certain I had a sufficient supply of materials to continue my DD2-IgG fusion work.

217. The experimental conditions of the digest are described at the bottom left corner of page 48 and reflect that following the digest, the samples were incubated overnight (“O.N.”)

at 37° (Celsius).

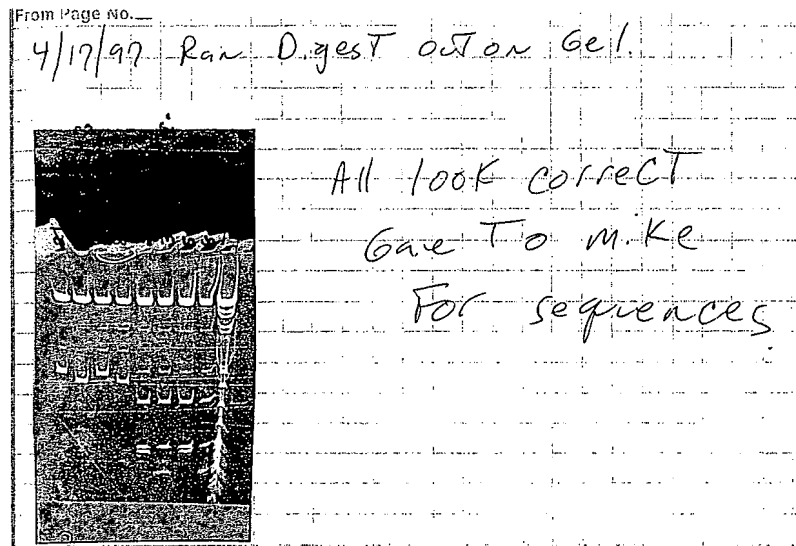
**24. Pages 49-50, Notebook 26119 (ADE-25)**

218. I conducted and recorded the activities described on pages 49-50 on April 17, 1997, as reflected by the dates recorded on that page.

219. My records reflect that I continued with the series of experiments I designed to obtain a DD.2-ECD-Flag fusion protein described previously on pages 47-48.

220. At the top of page 49, I wrote “Ran Digest out on Gel,” taped a photograph of the gel to the notebook page, and wrote “All look correct Gave to Mike for Sequences.” I do not recall the last name of “Mike” but he was a person who worked in the Genentech sequencing facility to whom I provided the digested materials for sequencing. These entries reflect that I ran the digests that I conducted on April 16, 1997, and described on page 48, on a gel, and the banding pattern I observed appeared to be correct based on the restriction enzymes I used for the digests.

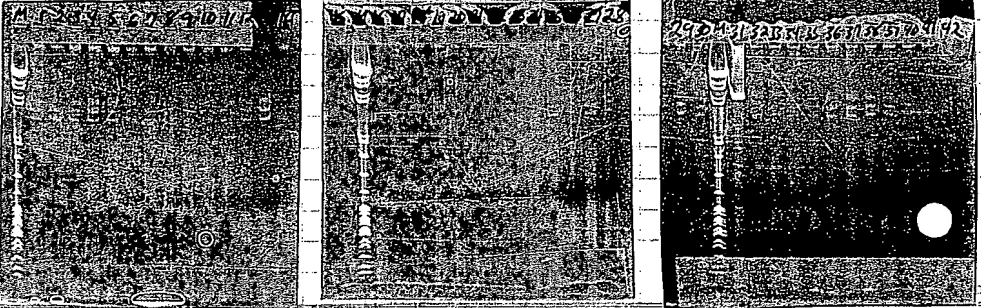
221. I labeled the photograph of the gel on page 49 with the numbers 21, 38, 56, 81, 21, 38, 56, 81 in the first eight lanes reading from left to right. These entries reflect that these were the DNA clones 21, 38, 56, and 81 that I used for the digests on April 16, 1997 and which I first selected on page 47, following PCR amplification of those sequences.



222. I further noted at the bottom of page 49 that "4/17/97 Set up PCRs of Transformation From 4/16 same protocol as on pg #46 except 5' primer SM.2; 3' primer uIgG P4." This experiment was a continuation of experiments directed to making DD.2-IgG fusion constructs. My entries reflect that I conducted PCR to amplify sequences using the above identified primers and DNA sequences from the JM109 cells transformed on April 16, 1997 and described at the middle of page 48. My activities are reflected in the image of page 49 set forth below. Following the PCR reaction, I ran the PCR products out on gels, photographs of which are attached to the bottom of page 49 and the top of page 50.



4/17/97 set up PCRs of Transformations From 4/16  
 same protocol as on pg # 46 except.  
 5' primer SM.2  
 3' primer U IgG P4  
 Then Ran on gels

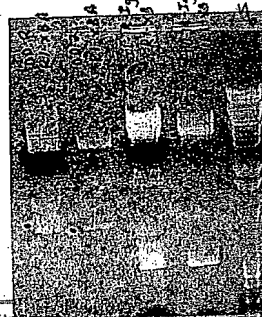


Witnessed & Understood by me,	Date	Invented by	Date
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223. I noted at the center of page 50 that “#31 looks correct pick template preps put on wheel O.N.” These entries reflect that I believed the PCR products loaded in lane #31 had the expected DD.2-IgG intermediate fusion insert. I selected clone number 31 and incubated the bacterial cells in LB broth supplemented with carbenicillin and K07 phage on a rotating wheel overnight in the warm room to grow phage containing single stranded DNA of the DD.2-IgG intermediate.

224. At the bottom of page 50, I made the following entries:

4/17/97 Took Backup Digests & ran a gel  
 cut out Bands marked.  
 cleaned up with a quick  
 Gel extraction kit.  
 + set up ligations  
 Rapid Ligation K.I.T.  
 SL + 1 (+ Frag)  
 Trans Formed into JM109s



Witnessed & Understood by me,	Date	Invented by	Date
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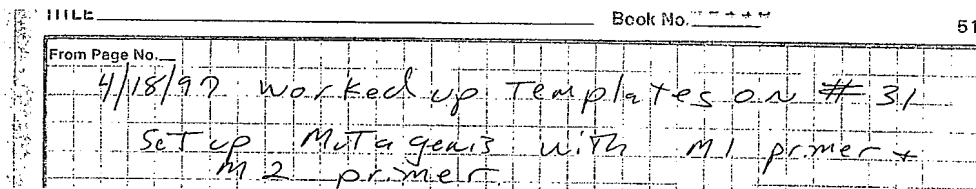
225. My entries reflect that I ran the “Backup Digests” I described at the bottom of

page 48 on a gel, and purified the DNA corresponding to bands of the expected size using a Qiaquick gel extraction kit. I then used a rapid ligation kit to ligate the “Backup” fragments into vectors and transformed JM109 bacterial cells (“JM109s”).

**25. Pages 51-54, Notebook 26119 (ADE-25)**

226. I conducted and recorded the activities described on pages 51-54 on April 18, 1997, as reflected by the dates recorded on that page.

227. My records reflect that I continued with the experiments described on pages 48-50 directed to making DD.2-IgG fusion constructs. Specifically the work I described on page 51 is a continuation of my work with “#31” DNA I previously identified as having the correct banding pattern for a DD2-IgG insert. The entry I made at the top of page 51 reflects these activities.



228. My entry “worked up templates on #31” reflects that I made single stranded DNA from the number 31 clone I previously described as having the DD.2-IgG insert, using K07 phage. I isolated the bacterial supernatant having the phage DNA, and isolated single stranded phage DNA which had the number 31 DNA incorporated therein.

229. I then used the “M1” and “M2” primers to make double stranded DNA from the single stranded phage DNA. This process spliced together the desired DD.2 and IgG sequences resulting in a DD.2-IgG fusion construct. I ligated the DNA into plasmids, transformed and plated JM109 cells, conducted filter lifts, and selected for positive clones.

230. Along the left side of page 51 I recorded the experimental conditions under which I conducted the mutagenesis experiments and are reflected in the image of the portion of page 51 below.

TITLE \_\_\_\_\_

From Page No. \_\_\_\_\_

4/18/97 worked up Temp  
 Setup Mutagenesis w/ T.  
 M2 primer.

Kinations  
 1  $\mu$ L oligo  
 1  $\mu$ L 10x CB  
 1  $\mu$ L 10mM ATP  
 1  $\mu$ L PAK  
 6  $\mu$ L dH<sub>2</sub>O  
 37° 45 min.

1  $\mu$ L K. nased oligo  
 1  $\mu$ L Template.  
 1  $\mu$ L annealing Buffer  
 6  $\mu$ L dH<sub>2</sub>O

5 min 68°  
 30 min 37°  
 5 min RT  
 Spin Down.  
 Add  
 1  $\mu$ L synthesis Buffer  
 1  $\mu$ L ligase  
 1  $\mu$ L T4 polymerase

5 min RT  
 2-4 hrs 37°

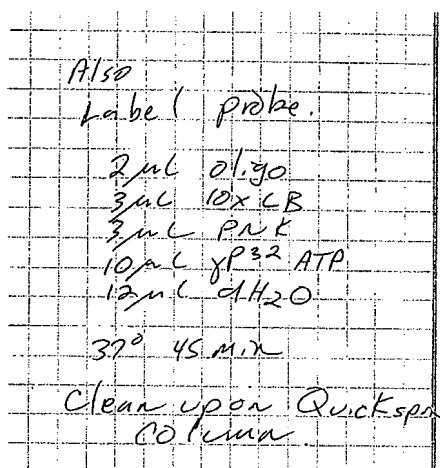
Add 90  $\mu$ L dH<sub>2</sub>O  
 Transformed into JM109s.

4/19/97 picked Filters.

231. As reflected in the entry above, after I completed the mutagenesis experiments, I transformed the newly ligated vectors carrying the mutagenized DNA into JM109 bacterial cells, placed filters over the transformed bacteria, and picked the filters from the JM109 transformants on April 19, 1997, as reflected by the entry "4/19/97 picked filters."

232. On the right side of page 51, I noted "Also label probes," reflecting that I radiolabeled probes which I used to probe the filters which were placed upon the transformed JM109 cells to detect positive colonies containing the DNA insert. I recorded my activities

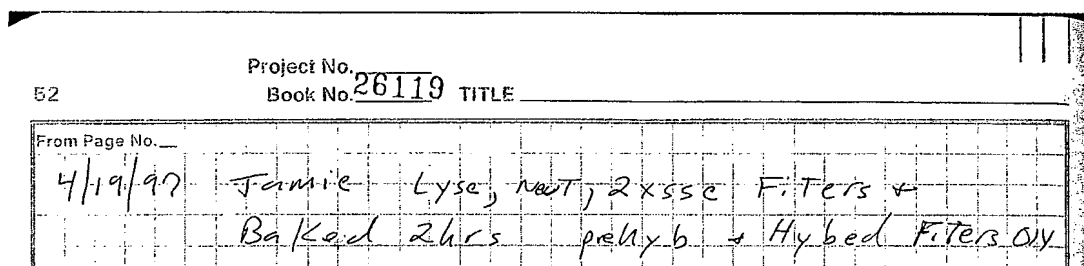
directed to making the radiolabeled probe on the right side of page 51 and is set forth in the image from page 51 set forth below.



Also  
label probe.  
2  $\mu$ L 0.1 gO  
3  $\mu$ L 10x CB  
3  $\mu$ L PNE  
10  $\mu$ L  $\gamma$ P32 ATP  
12  $\mu$ L dH<sub>2</sub>O  
37° 45 min  
Clean upon Quickspin  
column

233. My entries on pages 52-54 reflect that I continued working on the experiments I first described on page 51.

234. On April 19, 1997, I made several entries at the top of page 52, as reflected in the image below:



Project No. \_\_\_\_\_  
Book No. 26119 TITLE \_\_\_\_\_  
52  
From Page No. \_\_\_\_\_  
4/19/97 Jamie Lyse, nat, 2x55c Filters +  
Backed 2 hrs prehyb + Hybed Filters only

235. My entries reflect that Dr. Sheridan ("Jamie") prepared the filters I picked (described on the bottom of page 51) for hybridization with radioactive probes to select for positive clones. The probes I used were labeled M1 and M2 probes corresponding to the primers I used when I conducted the mutagenesis experiments. Dr. Sheridan incubated the filters with the probes overnight ("ON").

236. On April 20, 1997, I made several entries at the top of page 52, as reflected in the image below:

4/20/97 washed filters  
 1x 6xssc RT  
 2x 0.5xssc 37°  
 2x 0.2xssc 37°  
 put up for exposure. Below.  
 picked colonies as min. screens  
 M1 3, 6, 49  
 M2 7, 17, 29

237. My entries reflect that I washed the filters that Dr. Sheridan prepared to remove unbound or non-specifically bound probe from the filters. I placed autoradiographic film over the filters and allowed the film to expose. The radioactive M1 or M2 probes would expose the film in spots where bacteria expressed the corresponding M1 or M2 primer sequence. I included the exposures in a pocket I pasted onto page 52.

238. I identified colonies 3, 6, and 49 as positive for the M1 probe, and colonies 7, 17, and 29 as positive for the M2 probe.

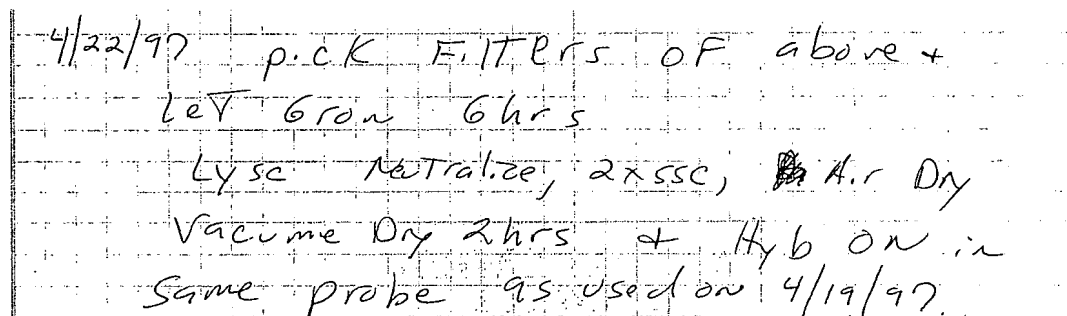
239. At the top of page 53, I made several entries, as reflected in the image below:

Project No. 26119  
 Book No. 53  
 TITLE  
 From Page No.  
 4/21/97 micro screen M1 3, 6, 49  
 M2 7, 17, 29  
 1st three steps of min. screen  
 + Transform into JM 1095  
 let Grow O/V

240. My entries reflect that on April 21, 1997, I conducted a "microscreen" of colonies designated M1 – 3, 6, and 49 and M2 7, 17, and 29. These colonies correspond to those I identified and described on page 52 which contained DNA encoding a DD.2-IgG fusion construct.

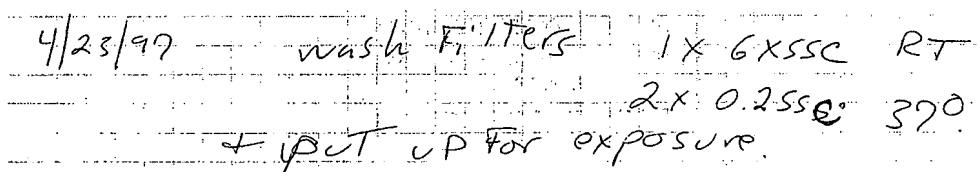
241. I noted that a "microscreen" is the "1<sup>st</sup> three steps of mini screen + transform into JM109s and grow ON." A microscreen is the alkaline lysis portion of a miniscreen without the following clean up.

242. On April 22, 1997, after conducting the microscreen on April 21, 1997, I picked filters from the microscreen and prepared the filters for hybridization using the same probe I used on the screens I conducted on April 19, 1997, and described on page 52. My activities are reflected in the image of the notebook page below.



4/22/97 p.c.k. FILTERS OF above +  
let grow 6 hrs  
lyse Neutralize, 2xSSC, ~~in~~ A.r Dry  
Vacume Dry 2hrs + Hyb ON in  
same probe as used on 4/19/97.

243. Following incubation of the filters overnight with the radioactive probe, on April 23, 1997, I washed the filters and exposed autoradiographic film to the filters to identify colonies containing DNA that hybridize to the M1 or M2 probes. The positive colonies I identified I designated "M1 3.4 and 6.31" and "M2 7.2 and 29.3." The first number in the designation corresponds to the number I identified from the first screen (e.g., M1 3 and 6) and the second number (e.g., .4 or .31) corresponds to the colony number from the microscreen. I included images of the filters in a pocket taped into page 53 of my notebook. My activities are reflected in the entries on page 53 reproduced below.



4/23/97 wash FILTERS 1x 6xSSC RT  
2x 0.2SSC 370  
+ PUT UP for exposure.

244. I then picked the positive colonies and made plasmid preparations from the

positive clones. I sent the plasmid preparations for sequencing. I later used these plasmids to transfect 293 cells as described on page 5 of my notebook 27505.

From Page No. \_\_\_\_\_

4/23/97 prcted plasmid preps of

M1	3.4	+	6.31
M2	7.2	+	29.43

**26. Page 1, Notebook 27505 (ADE-26)**

245. I conducted and recorded the activities described on page 1 on April 18, 1997, as reflected by the dates recorded on that page.

246. My records reflect that I set up transfection experiments using 293 cells and the DNA encoding the Apo-2-ECD Flag. I note that when describing the DNA to be used in these transformation experiments, I made the following notation "200 ul Prk5 Apo-2 (DD.2) ECD Flag" reflecting that I knew that the DD.2 DNA was now being designated "Apo-2." An image of my notebook page reflecting these entries is set forth below.

Project No. \_\_\_\_\_  
Book No. **27505**

TITLE \_\_\_\_\_

From Page No. \_\_\_\_\_

4/18/97 set up Tx in 2935 cells.  
20 plates  
Fed 12:00 noon

Tube A	Tube B
10mls 1:10 TE	10mls 2x HBS
20µL VARNA (DD.2)	
200µL Prk5 Apo-2 ECD Flag	
1ml 2.5M CaCl <sub>2</sub>	

Mix A into B  
wait 5 min + plate 1ml/plate.  
DNA on cells 3:30 pm.

247. My entries reflect that I also described the reagents I used in "Tube A" and "Tube

B”, that I mixed the contents of tube A into tube B, and that I plated the DNA onto the cells.

248. On April 19, 1997, I fed the cells contained on the plates I prepared on April 18, 1997 “serum free media.” I recorded this activity at the middle of page 1.

249. On April 21, 1997, I noted that I “Harvested 40mls to set up expt” which reflects that I harvested 40 milliliters of the supernatants from the cells I transfected with the Apo-2 ECD-Flag DNA.

250. On April 22, 1997, I noted that I “Harvested rest of material [supernatants] + Gave to Bob for purification” which reflects that I harvested the remainder of the supernatant from the cells I transfected with the Apo-2 ECD Flag DNA and gave the material to Bob Pitti, a Senior Research Associate working in Dr. Ashkenazi’s lab, for purification.

**27. Pages 2-3, Notebook 27505 (ADE-26)**

251. I conducted and recorded the activities described on pages 2-3 on April 21-23, 1997, as reflected by the dates recorded on that page.

252. My records reflect that I set up and conducted a NFκB assay using the Apo-2 receptor.

253. On April 21, 1997, I made several entries on page 2, as reflected in the image below:



Project No. \_\_\_\_\_ TITLE NFκB Assay

Book No. 27505

From Page No. \_\_\_\_\_

4/21/97 Electroporated HeLa.s  
 set up 2 sets of each + combined to 1 plate  
 100ng Plasmid / set + lmg VARNNA 60mm

Plate #	DNA
1	pIKS
2	Apo-2
3	DR4
4	Apo-3
5	TNFR1

4/22/97 Set up NFκB Assay.  
 As usual.

Electroporated  
 1:00 p.m.  
 4:00 p.m. Fed + washed  
 cells  
 Added  
 ZVAD. 100μM  
 (6μl of 50mM stock into  
 3mls media.)

254. The entry I made at the top of page 2 reflect that the work I conducted was directed to a "NFκB assay." The entry "4/21/97 Electroporated HeLa.s" reflects that I transfected HeLa cells with the DNA listed on the page. I listed the DNA I used for the transfection to the right of the corresponding plates numbered 1-5 toward the top of page 2.

255. I noted toward the right side of page 2 that I "added ZVAD 100uM" to the transfected cells. As described earlier, ZVAD is a caspase inhibitor and I used ZVAD to evaluate the role of caspases in Apo-2 receptor signaling of apoptosis.

256. On April 22, 1997, I noted on page 2 that I "Set up NFκB Assay As usual." Below that entry I described the reagents that I added to the sets of tubes I called A, B, and C. I noted that I incubated the samples with cold probe and NFκB antibody to reduce non-specific binding and to induce a mobility shift, respectively, as described earlier.

257. Following incubation, I loaded two gels in the order described at the bottom of page 2 and exposed the gels overnight ("ON"). My activities are set forth in the image of page 2 below. I taped a pocket onto page 2 which contains an image of the results from this NFκB assay.

4/22/97 SET up NFkB Assay.  
As usual.

ZVAD. 100  $\mu$ M.  
(5  $\mu$ L OF 50 mM STOCK IN 3 mL MEDIA.)

A's had nothing added.  
B's had 5  $\mu$ L OF Cold Probe Added  
C's had 3  $\mu$ L OF  $\alpha$  NFkB AB Added. 15 min AFTER START OF RXN.

Gel loading order  
Gel 1  
1A/2A/3A/4A/5A/1C/2C/3C/4C/5C/  
4/22/97 expose O.N.

Gel 2  
1B/2B/3B/4B/5B

258. The results from the gels I ran reflect that NFkB activity increased in the presence of the caspase inhibitor ZVAD.

**28. Page 4, Notebook 27505 (ADE-26)**

259. I conducted and recorded the activities described on page 4 on April 21, 1997, as reflected by the dates recorded on that page.

260. My records reflect that I conducted binding experiments using the Apo-2-ECD Flag fusion protein and evaluated whether the fusion protein binds Apo-2-ligand (Apo-2-L).

261. At the top of page 4 I wrote "Does Flag ECD Apo-2 bind Apo-2L?." After I obtained the results from this experiment I wrote "yes!!!."

4	Project No. _____	Does Flag ECD Apo2 Bind Apo2L? yes!!!
	Book No. <b>27505</b>	TITLE _____
	From Page No. _____	
	4/21/97	

262. The purpose of this experiment was to evaluate whether Apo-2 ligand binds to the Apo-2 receptor ECD-flag fusion protein.

263. Along the left side of page 4, I listed the tube numbers and the contents of the tubes that I used to conduct the binding experiments. The tubes contained either culture media alone ("SF Media"; tubes 1, 5, 9, 13), media containing the Apo-2 fusion protein ("Apo-2

media"; 2, 6, 10, 14), or media containing the Apo-2 fusion protein and Apo-2-ligand (tubes 3, 7, 11, 15), or SF media and Apo-2L (tubes 4, 8, 12, 16).

264. Under the "IP" (Immunoprecipitation) column I listed "Ni Resin" (Nickel Resin) as the reagent I used to precipitate receptor-ligand complexes through the ligand from tubes 1-8. I noted that I used " $\alpha$ -Flag resin" for tubes 9-16 reflecting that I used an anti-Flag antibody bound to a resin to precipitate bound receptor ligand complexes through the receptor from tubes 9-16. I expected the antibody to bind the Flag portion of the Apo-2-ECD-Flag fusion protein which allowed for isolation of receptor-ligand complexes. The nickel resin precipitated the Apo-2L via a poly-His tag fused to the Apo-2L I used. The image below reflects my entries from page 4.

Project No. 27505 TITLE Does Flag ECD Apo2 Bind Apo2L? yes!!!

From Page No. 4/21/97

Tube #	use SMLs media	use Apo2L SML (mg/ml Final)	IP
1	SF media		Ni Resin
2	Apo2 media		"
3	"	+ Apo2L SML (0.84 mg/ml)	"
4	SF media	"	"
5	"		"
6	Apo2 media		"
7	"	+ Apo2L SML (0.84 mg/ml)	"
8	SF media	"	"
9	SF media		$\alpha$ Flag Resin
10	Apo2 media		"
11	"	+ Apo2L SML (0.84 mg/ml)	"
12	SF media	"	"
13	"		"
14	Apo2 media		"
15	"	+ Apo2L SML (0.84 mg/ml)	"
16	SF media	"	"

265. After I prepared the experiments, I incubated the media (e.g., Apo-2 media) with Apo-2-ligand for thirty minutes at room temperature on a rotator. Following the "media" incubation, I added resin to the media (either nickel resin or the  $\alpha$ -Flag resin) and incubated the reaction tubes for another 1.5 hours. The image below reflects my activities.

Incubate media with Apo2L 30 min RT		0° rotator
Add 25 µl of washed Resin (in TBS) 1.5 hr		4° rotator
Wash Resin 4X TBS		
Add SB + DTT Boil 10' + Load gel		
Gel #1	M/1/2/3/4/9/10/11/12	
Gel #2	13/14/15/16/5/6/7/8/M	
+ Blot to nitrocellulose		put in 5% milk to block O.N.
To Page No. 6		
Witnessed & Understood by me,	Date	Invented by

266. My entries reflect that following the resin incubation, I washed the resin with buffer, added sample buffer and dithiothreitol, and boiled the resin for ten minutes to release bound receptor-ligand complexes from the resin. I loaded the samples on a gel, in the order described at the bottom of the page, and blotted the gels to nitrocellulose filters. I put the filters in a 5% milk solution to block any non-specific binding of antibody to the filter paper. I wrote a "6" at the bottom of page 4 next to the "To Page No.\_\_\_\_" entry indicating that page 6 has additional information relating to these experiments.

267. My work from this experiment is continued on page 6 of notebook 27505.

**29. Page 5, Notebook 27505 (ADE-26)**

268. I conducted and recorded the activities described on page 5 on April 21, 25, 26, 28, and May 2, 1997, as reflected by the dates recorded on page 5.

269. My records reflect that I set up plates of 293 cells to be used for transformation experiments "later in the week." The experiments that I conducted later that week on April 25, 26, and 28 involved transfecting cells with Apo-2-IgG fusions or Apo-2-ECD-Flag fusions.

270. On April 25, 1997, I transfected 293 cells with DNA encoding an Apo-2-IgG fusion protein and cells with DNA encoding an Apo-2-ECD-Flag fusion protein as reflected in my notebook entries, an image of which is reproduced below. I described preparation of these

constructs on page 54 of my notebook 26119.

2935 cells For TX.

Project No. \_\_\_\_\_  
Book No. 27505

TITLE \_\_\_\_\_

From Page No. \_\_\_\_\_

2935 100mm Dishes

4/21/97 set up 160 plates For TX Later in the week.

4/25/97 Fed cells 12:00 noon.

100 plate TX with Apo-2-IgG  
60 plate TX with Apo-2-EC0-Flag.  
+ 1ug VARNA / 10ug DNA / plate

DNA on cells 4:00 pm

271. On April 26, 1997, I fed the cells, which I transfected on April 25, serum free culture media.

272. On April 28, 1997, I noted that "took samples + gave to Assay services for Fc elisa" which reflects that I took supernatant samples from the cells I transfected on April 25, 1997 and provided the supernatants to Genentech's Assay Services group to conduct an ELISA analysis to detect Apo-2-IgG fusion proteins. I attached the assay request form to page 8 of my notebook.

273. On April 28, 1997, I also "harvested s.f. sup of IgG plates + Gave to Bob to purify" which reflects that I harvested the supernatant from plates containing cells transfected with the Apo-2-IgG fusion DNA and provided the supernatant to Bob Pitti so that he could conduct experiments to purify the Apo-2-IgG fusion protein from the supernatant. I also re-fed the plates with fresh S.F. media.

274. On May 2, 1997, I "harvested sups from plates" which reflects that I harvested the supernatant from the second feeding of the plates I transfected on April 25, 1997.

275. I recorded my activities on page 5 of my notebook, an image of which is set forth below.

4/26/97 Fed cells Serum Free media  
4/28/97 <sup>am</sup> Took samples & Gave to Assay services  
For FC elisa. pg# 8  
4/28/97 <sup>pm</sup>  
Harvested S.F. sup of IgG plates  
& Gave to Bob to purify.  
Re Fed plates with new S.F. media  
5/2/97 Harvested sups from plates.

**30. Page 6, Notebook 27505 (ADE-26)**

276. I conducted and recorded the activities described on page 6 on April 22, 1997, as reflected by the dates recorded on that page.

277. My records reflect that I continued my experiments that I started on April 21, 1997, and described on page 4.

278. Across the top of page 6, I entered a "4" next to the "From Page No. \_\_\_\_" entry reflecting that the activities were continued from page 4. I also wrote "4/22/97 wash blots 1 x TBST" reflecting that I washed the immunoblots I prepared on April 21 with tris-buffered saline containing tween ("TBST").

279. After I washed the blots from gels 1 and 2, I incubated the blots with either "3ul  $\alpha$ -Flag (M2)", (blot from gel 1), which is a Flag-specific antibody, or "2ul polyclonal #3  $\alpha$ -Apo-2L", (blot from gel 2) which is an Apo-2L specific polyclonal antibody. The  $\alpha$ -Flag primary antibody was derived from mouse, whereas the  $\alpha$ -Apo-2L antibody was derived from rabbits.

280. After incubation of the blots from gels 1 and 2 with the primary antibodies, I

washed the blots to remove non-specific binding, and then incubated the blots with secondary antibodies. I incubated the gel 1 blot with a sheep anti mouse antibody, which is conjugated to HRP. I incubated the gel 2 blot with an anti-rabbit antibody, which is also conjugated to HRP. HRP allows for visualization of specific binding of the secondary antibody to the primary antibody.

281. I then exposed the blots and developed the films. The blots and films are inserted in a pocket that I taped into the bottom of page 6.

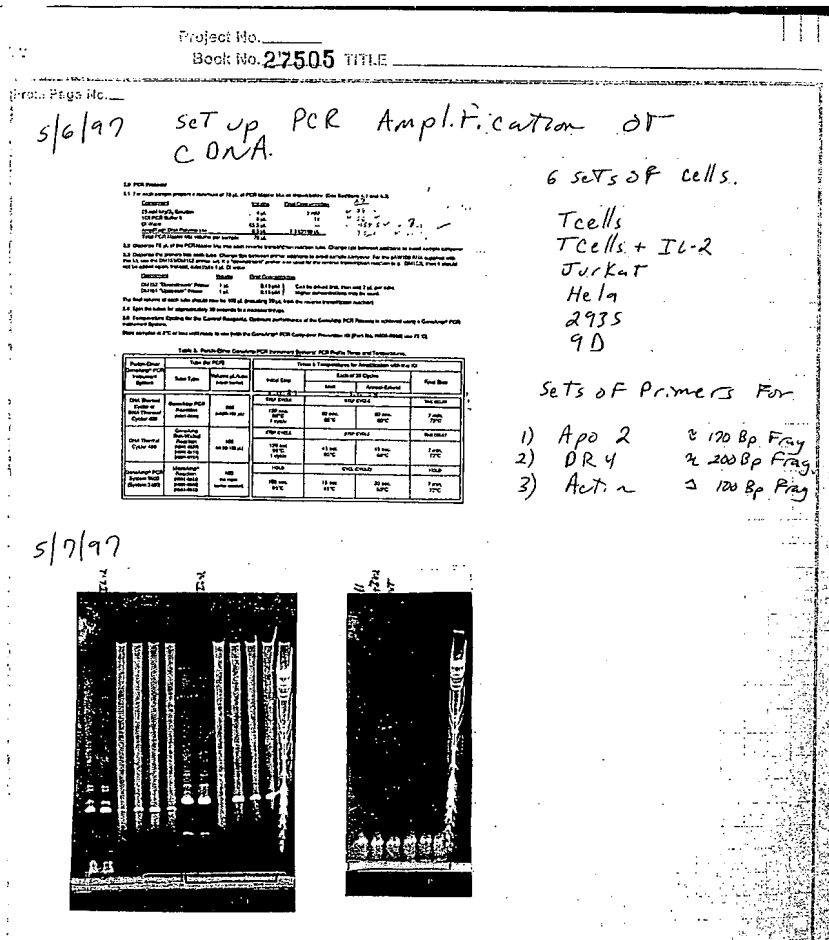
282. The results I observed in the gels reflect that Apo-2L and the Apo-2-ECD Flag fusion protein were detected as a complex reflecting that Apo-2L bound to Apo-2-ECD-Flag.

**31. Page 18, Notebook 27505 (ADE-26)**

283. I conducted and recorded the activities described on page 18 on May 6, 1997, as reflected by the dates recorded on that page.

284. My records reflect that I set up a PCR amplification experiment to amplify Apo-2 DNA in cDNA libraries derived from various cell types. The purpose of this experiment was to identify cell types expressing the Apo-2 receptor.

285. On May 7, 1997, I ran the PCR products on a gel and I taped photographs of the gels onto page 18 of my notebook. The image of my notebook entries set forth below reflects these activities.



#### D. Activities Conducted by Maya Skubatch

286. As described in an earlier section of this declaration, Maya Skubatch was a temporary research associate in the Ashkenazi lab.

287. While working under my immediate direction and supervision, Ms. Skubatch conducted experiments relating to the characterization of the Apo-2 receptor. I describe these experiments below.

288. I reviewed Ms. Skubatch's notebooks 26577 and 27236 prior to providing the descriptions below.

289. All dates prior to March 17, 1997 have been redacted.


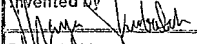


1. Pages 76-77, Notebook 26577 (ADE-27)

290. Ms. Skubatch conducted and recorded the activities described on pages 76-77 prior to March 17, 1997, as reflected by the dates recorded on those pages.

291. Based on subsequent entries in Ms. Skubatch's notebooks, the purpose of these experiments was to obtain a probe for use in Northern blot analyses.

292. At the middle of page 76, Ms. Skubatch made the following entries:

<u>DD.2 Receptor</u> Purify (R1-H3) fragment using GigaGene Agarose technique			
DD.2 DNA 80 ng/ml			
			
Label with probe			
① 3 ul DD.2 (R1-H3) 2 ul dH <sub>2</sub> O 10 ul primers (brn cap) 100° 5'	② 10 ul dCPT Buffer (blk cap) 1 ul Klenow (grn cap) 5 ul dCPT α 37° 15'	on column 25K 50 ul ss Salmon Sperm 5' 100K add to hybridization	
Witnessed & Understood by me,	Date	Invented by 	Date

293. The entries under "DD.2 Receptor" reflect that Ms. Skubatch purified EcoR1 and HINDIII digested DD.2 ("R1-H3") fragments.

294. The entries toward the bottom of page 76 under the "Label with probe" entry reflect that Ms. Skubatch labeled the digested DD.2 fragments to make a probe which she used to probe blots in a Northern experiment. She listed the procedures that she followed to label the probe at the bottom of the page and the reagents used to do so.

295. Toward the middle of page 77, Ms. Skubatch made the following entries:

Blots, mRNA

Strip blots:  
 boil 1L dH<sub>2</sub>O in 2L flask w/ 5ml 20% SDS.  
 dry blots on paper, air dry.  
 use expresshyb soln' (at 65°) and pre blots 30'  
 transfer blots to new plate w/ express hyb and add hot probe.  
 (see p. 76)  
 Hyb O.N. 65°

USER: 1	ID: 32P	COMMENTS: 32P	
PRESET TIME: 2.00	HA: NO	SAMPLE REPEATS: 1	
PRINTER: STD	SCR: NO	REPLICATES: 1	
RS232: OFF	RCM: YES	MULTIPLIER: 1.0000	
ISOTOPE 1: 32P	%ERROR: 0.00	BKG. SUB:	0
SAM NO	POS	TIME MIN	32P CPM %ERROR RCM E'
1	1-1	2.00	97819.54 0.45 0.01

50,000,000 cpm / 50 ml

296. The above entries reflect that Ms. Skubatch stripped nitrocellulose blots in preparation for conducting a Northern analysis. I provided her with the blots. The entries in Ms. Skubatch's notebook suggest that she prepared the blots according to the protocol described in the entry above, which included incubating the blots in an express hybridization solution, prehybridizing the blots for thirty minutes, transferring the blots to a new plate and adding a radiolabeled probe (which she described on page 76) that would bind to sequences on the blots having homology to the DD.2 sequence. Ms. Skubatch allowed the probe to hybridize to the blot overnight at 65°C as reflected by the entry "Hyb O.N. 65°."

297. At the bottom of page 77, Ms. Skubatch taped the output from the scintillation counter measuring the radioactivity of the probe that she used to probe the blots.

## 2. Pages 78-79, Notebook 26577 (ADE-27)

298. Ms. Skubatch conducted and recorded the activities described on pages 78-79 prior to March 17, 1997, as reflected by the dates recorded on those pages.

299. The work described on page 78 is a continuation of the Northern blot experiments

described on page 77. Ms. Skubatch noted at the top of the page “Northern Blots Wash” reflecting that she washed the blots she described on page 77. The purpose of washing the blots was to remove unbound radioactive probe from the blots. She listed the specific solutions she used to conduct the washes at the top of the page.

300. Following the wash, Ms. Skubatch exposed the blots to a phosphorimager which is a device that detects the radiation emitted from the  $^{32}\text{P}$  isotope incorporated into the probe she used to probe the blots.

301. Ms. Skubatch included the results from the Northern analyses in a pocket that she taped onto page 79. The results reflected that DD.2 mRNA was present in several tissue samples including fetal kidney, liver, and lung, and adult PBLs (peripheral blood leukocytes), colon, small intestine, ovary, testis, prostate, thymus, spleen, pancreas, kidney, skeletal muscle, liver, lung, placenta, and heart.

302. Ms. Skubatch conveyed these results to me on the same day that she obtained the results and I included a copy of the blots on page 56 of my notebook 26865.

### **3. Pages 82 and 84-86, Notebook 26577 (ADE-27)**

303. Ms. Skubatch conducted and recorded the activities described on pages 82 and 84-86 prior to March 17, 1997, as reflected by the dates recorded on those pages.

304. On page 82, Ms. Skubatch set up PCR experiments using transformants that I provided to her and PCR primers designated SM.2 and uIgG 4.

305. On page 84, Ms. Skubatch attached images of gels containing DD.2-IgG PCR products. Ms. Skubatch wrote “DD.2-IgG” at the top of page 84 reflecting that the samples were DD.2-IgG fusions. She made asterisks on sample numbers 6 and 20 reflecting that these samples had the proper banding pattern and that these samples would be used to make a larger scale preparation of the DNA in these samples.

306. Ms. Skubatch further noted at the bottom of page 84 “pick 6, 20 for 1L Prep.”

307. Toward the middle of page 85, Ms. Skubatch noted “DD.2-IgG” and noted below that entry “Maxi Prep #6, #20.” These entries reflect that she conducted a maxi-prep purification of the DNA constructs numbered 6 and 20, as shown on page 84, encoding a DD.2-IgG fusion protein.

308. At the bottom of page 85, Ms. Skubatch made the following entries reflecting her work directed to conducting radiation hybrid experiments:

DD.2/ Radiation Hybrid				
2.5 ul DNA	X100			
2.5 ul PCR Buffer (Clontech)	250		94° 3'	1 cycles
6.0 ul dNTP'S	600		94° 1'	60° 1' 72° 2' 35 cycles
0.35 ul P1 (DD-PCR 5')	25	1-78, 81-85		
0.5 ul P2 (DD-PCR 3')	25			
0.25 ul T4 Polymerase	25	84	G3/RM	+
13 ul dH <sub>2</sub> O	1300	85	G3/A3	-
25 ul Total				
To Page No. _____				

309. I asked her to conduct these experiments and the purpose of conducting the radiation hybrid experiment was to map the chromosome to which the DD.2 DNA was localized. Ms. Skubatch used a commercially available kit from Research Genetics to conduct the radiation hybrid experiments. She listed the reagents she used in the experiments at the bottom of page 85 as reflected in the image above.

310. Ms. Skubatch noted at the top of page 86 “Run Hybrid PCR on gel” and taped photographs of gels she ran to page 86. The gels reflected a banding pattern which was translated into a computer code and submitted to the Stanford Mapping facility. This was the end product of the hybrid experiment.

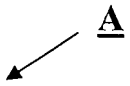
311. The end product of the hybrid experiments produced a DNA marker that Ms. Skubatch designated “dd.2.” She provided the results and information from her experiment to me and we sent the information she provided to me to the Radiation Mapping Facility at Stanford

University to determine the chromosome localization of the dd.2 marker.

312. Ms. Skubatch attached a printed page to page 85 of her notebook that she received from me reflecting that the dd.2 marker mapped to chromosome 8. An image of a portion of the printout of page 85 reflecting the chromosome 8 localization is set forth below at arrow "A."

The calculation results were:

<u>submitted marker</u>	<u>linked marker</u>	<u>LOD</u>	<u>cR_10000</u>	<u>chrom</u>
Reporting best lod >= 6.0				
dd.2	D8S481	11.054567	9.90	8



#### **4. Pages 88-91 Notebook 26577 (ADE-27)**

313. Ms. Skubatch conducted and recorded the activities described on pages 88-91 on March 17 and 18, 1997, as reflected by the dates recorded on those pages.

314. The activities that Ms. Skubatch recorded on pages 88-91 were directed to conducting PCR experiments to amplify sequences from DNA libraries derived from various fetal tissue sources such as kidney, lung, and small intestine. I asked her to conduct these experiments to confirm that the DD.2 sequence that had been identified and cloned in Dr. Ashkenazi's lab was present in multiple tissue sources and to look for alternative splice forms, if any, in the libraries.

315. The entries on page 88 reflect that Ms. Skubatch set up PCR experiments using a variety of libraries from different tissue sources. I gave Ms. Skubatch the libraries. She also recorded the reagents that she used in the PCR experiment (upper right corner of image below) and the primer sets as recorded under the "Primer sets" entry on the right side of the page. The primers having a "prk" designation (*e.g.*, prk Race 1) hybridize to sequence contained in the prk5 vector; the primers having a "DD" designation hybridize to a sequence within the DD.2 sequence we sought to amplify; and the "SM.1" primer hybridized to the 5 prime region of the

extracellular domain of the DD.2 sequence. She designated each primer set A-F. An image of page 88 is set forth below.

PCR	Prk5	DD Race	
Sample	Library		1. ul cDNA library 12 ul dNTPs 5 ul PCR buffer 0.5 ul P1 0.5 ul P2 31 ul dH <sub>2</sub> O
1-6	Hu fetal lung → 2500		
7-12	Hu fetal lung 1.3-2.5		
13-18	Hu fetal lung 0.6-1.3		
		Primer sets	
		A prk Race 1	DD Race 3
		B prk Race 1	DD Race 2
19-24	Hu fet. Sm. intes → 2500	C SM 1	DD Race 3
		D SM 1	DD Race 2
25-30	Hu fet. small intes 1.3-2.5	E prk Race 2	DD Race 3
		F prk Race 2	DD Race 2
31-36	Hu fet. small intes 0.6-1.3		
		94° 1' 60° 1' 72° 2' 35 cycles	
37-42	fetal kidney prk 5b → 2.8 kb		
43-48	fetal lung → 2.8 kb		
49-54	fetal kidney prk 5 → 2.8 kb		
55-60	fetal lung → 2.8 kb		
61-66	fetal liver prk 5 1:66		

316. The entries under “Sample” reflect the sample numbers corresponding to the libraries from each tissue source listed immediately to the right under the column labeled “Library.” There were 66 samples in this experiment numbered 1 through 66.

317. The entries under the “Primer sets” section reflect that Ms. Skubatch conducted 35 cycles of PCR. The PCR reaction was run at 94° for 1 minute, then 60° for 1 minute, followed by 72° for 2 minutes (“94° 1’ 60° 1’ 72° 2’’”).

318. Ms. Skubatch ran the PCR products on gels and taped photographs of the gels to page 89 of her notebook. The images are labeled A-F under each corresponding tissue designation. The sample numbers for each tissue source (e.g., 1-6 for Human Fetal Lung library)

correspond to the sample numbers labeled A-F on the photographs of the gels that she attached to page 89 (e.g., A-F for “Fetal Lung >2500”). The A-F designations reflect the particular primer set used for that particular sample in accord with the designations she made at the right side of page 88. For example, she used primer set “A” for sample 1. Primer set A corresponds to primers “prk Race 1 and “DD. Race 3.”

319. On March 18, 1997, Ms. Skubatch continued with the PCR experiments she began on March 17, 1997, and described on page 88. She continued her activities on pages 89, 90, and 91.

320. At the bottom of page 89, Ms. Skubatch wrote “Re-PCR 1  $\mu$ l aliquot (2<sup>nd</sup> Round)” and listed a number of samples and numbers below that entry. These entries reflect that she identified several PCR products from the experiments she conducted on March 17 and that she conducted additional PCR experiments using the PCR products from the listed sample numbers and different PCR primers. Ms. Skubatch listed ten samples at the bottom of page 89 and continued the list to the top of page 90. The image below reflects these entries.

March 18, 1997 - Tuesday									
Re-PCR 1 $\mu$ l aliquot (2 <sup>nd</sup> Round)									
#10	1	#10	(SM.2 DD.race 1)	Hfslung	6	#39	(SM.2 DD.race 2)	f k <2.8	
	2	#22	(SM.2 DD.race 1)	Hfslung	7	#39	(SM.2 DD.race 1)	f k <2.8	
	3	#23	(SM.2 DD.race 2)	Hfslung	8	#44	(prk race 2 DD.race 1)	f k <2.8	
	4	#27	(SM.2 DD.race 1)	Hfslung	9	#49	(prk race 2 DD.race 2)	f k >2.8	
	5	#38	(prk race 2 DD.race 1)	f k <2.8	10	#49	(prk race 2 DD.race 2)	f k >2.8	No cont

321. The consecutively listed numbers in the image above reflect the sample numbers for the PCR experiments. Immediately to the right of those numbers are the numbers (preceded by a # sign) of the PCR products from the first round of PCR (e.g., sample “1” corresponds to “#10”). The next entry, reading left to right, reflects the PCR primer designations used for that particular sample. (e.g., sample 1, #10, used primers designated SM.2 and DD.Race1). The last

entry, reading left to right, reflects the tissue origin of the DNA library from which the PCR products were originally isolated (*e.g.*, sample 1, #10, was isolated from a human fetal lung library having the designation “Hufe lung 1.3-2.5). The numbers following the library designation (*e.g.*, 1.3-2.5) reflects the size of the cuts made in the library DNA (in kilobases) to make the libraries.

322. Ms. Skubatch listed the remainder of the 16 samples she prepared for this experiment at the top of page 90 following the same nomenclature format as that provided at the bottom of page 89.

323. Ms. Skubatch recorded the PCR reaction conditions and the reagents she used in the PCR reactions at the middle of page 90 under the entry “PCR Reaction”. On the bottom left side of page 90, underneath an image of a gel that she taped into her notebook, she also listed ten samples under the entry “Repeat” reflecting that she planned to repeat the PCR reaction for the samples listed. The samples she listed were 10, 22, 27, 39, 44, 49, 51, 58, 62 and 63.

324. The photograph of the gel Ms. Skubatch attached to page 90 contains four asterisks at sample numbers 11, 12, 13 and 15, which correspond to DNA from the initial PCR experiments corresponding to #51, 58, and 63. The asterisks reflect that the samples in these lanes had the expected banding pattern for the primers used in the PCR reaction.

325. At the top of page 91, Ms. Skubatch wrote “PCR” with the word “redo” directly below, reflecting that she was repeating the PCR reaction for the samples listed at the top of page 91. The samples she listed included those samples she listed at the bottom of page 90 and were sample numbers 10, 22, 27, 39, 51, 58, and 63. As before, reading from left to right, she listed: 1) a sample number (1-7); 2) a second number preceded by a pound sign (*e.g.*, #10) reflecting the sample obtained from the initial PCR experiment as described on page 88; 3) the source



designation from which the PCR product was derived (*e.g.*, human fetal lung); 4) and the primers used in the PCR reaction (*e.g.*, SM.1, DD.Race2).

326. Ms. Skubatch listed the reagents she used in the PCR reaction at the middle of the page and taped a labeled photograph of the gel she ran containing the PCR products from this experiment. The labels reflect the PCR products from samples numbered 10, 22, 27, 39, 51, 58 and 63, reading right to left. The results Ms. Skubatch obtained suggest that all samples except sample 10 had the expected PCR product corresponding to the DD.2 sequence.

#### **5. Pages 92-93 Notebook 26577 (ADE-27)**

327. Ms. Skubatch conducted and recorded the activities described on pages 92-93 on March 19, 1997, as reflected by the dates recorded on those pages.

328. At the top of page 92, Ms. Skubatch wrote “Run PCR on 6% TBE gel p. 91” reflecting that she ran the PCR products from page 91 on a gel. As mentioned above, she attached a photograph of the gel she ran to page 91.

329. Ms. Skubatch further noted at the top of page 92 “Purify fragments (11, 12, 13, 15) (2-7)” reflecting that she would: 1) purify fragments 11, 12, 13, and 15 from the second round of PCR experiments she conducted which are described on page 90 and highlighted by the asterisks on the photograph of the gel on page 90; and 2) purify samples 2-7 described at the top of page 91. She taped a “Technical Bulletin” from a Promega DNA purification kit which she used to purify the fragments described above.

330. At the top of page 93, Ms. Skubatch wrote “Quick Ligation of PCR into pGEMT” reflecting that she ligated the PCR products that she purified, as described on page 92, into a pGEMT plasmid vector. She listed the reagents used in the ligation on the top left side of the page and listed the sample numbers at the top of the right side of the page. Ms. Skubatch numbered the samples 1A through 10A and listed the original PCR designation from the

experiment on page 88 (e.g., “#22”) and the PCR primers she used to obtain the various purified PCR products to the right of each (e.g., “sm.1, race2”). The image below reflects these entries.

Project No. \_\_\_\_\_ Book No. 26577 93

TITLE \_\_\_\_\_

From Page No. \_\_\_\_\_

Quick Ligation of PCR into PGEMT

1 ul vector	SL	1A #12 (sm.1 race2)	
7 ul insert	2A #21 (sm.1 race2)	6A #63 (sm.1 race2)	
2 ul vial 2	3A #34 (sm.1 race2)	7A #51 (sm.1 race2)	
10 ul vial 1	4A #51 (sm.1 race2)	8A #51 (sm.1 race2)	
Mix	5A #38 (sm.1 race2)	9A #58 (sm.1 race2)	
1 ul vial 3	6A	10A #63 (sm.2 race2)	
5 min RT			

Transformations

20' 0"

45' 42"

2' 0"

45' 37" on wheel plate 100 ul on (NZYDT / IPTG)

pet.muAFO2L pet.muAFO2L vol. pat19f/r

Submit for sequencing AFO2L 10, 76, 82 ul. for

DD.2 lgt # 1 ul 1967

331. Following the ligation experiment, Ms. Skubatch transformed the newly ligated DNA into bacterial JM109 cells. This activity is reflected by the entry “Transformations” and the entries directly below that entry. Following incubation of the cells for “45' 37° on wheel” (45 minutes at 37 degrees Celsius), she plated one hundred microliters of the transformed cells on IPTG/X-GAL. IPTG induces expression of the lacZ gene which converts X-GAL to a blue stain. This system is used to confirm transformation of the cells. (Ms. Skubatch erroneously wrote “NZYDT/IPTG” in her notebook. This is not a reagent that she would have used in this experiment).

332. Ms. Skubatch also conducted and recorded new experiments on page 93. She made the following entries toward the bottom of page 93:

DD.2 196- # 44 1967

DD.2 - 5' PCR		Library	Primer	Rxn	X6
1	Human spleen	(SM.1	DD Race 3)	1 ul DNA	
2	"	("	DD Race 2)	1 ul P1	6 ul
3	Hu Thymus	("	DD Race 3)	1 ul P2	6 ul
4	"	("	DD Race 2)	10 ul Buffer	60 ul
5	Hu Genomic	("	DD Race 3)	24 ul 1.25 dNTPs	144 ul
6	"	("	DD Race 2)	1 ul Tq	6 ul
7	Hu Lung	("	DD Race 3)	62 ul H <sub>2</sub> O	372
8	"	("	DD Race 2)		
9	Hu Liver	("	DD Race 3)		
10	"	("	DD Race 2)		
11	fe Heart	("	DD Race 3)		
12	"	("	DD Race 2)		

94° 1' 60° 1' 72° 2' 3 cycles

Witnessed & Understood by me, \_\_\_\_\_ Date \_\_\_\_\_ Invented by \_\_\_\_\_ Recorded by \_\_\_\_\_

333. Ms. Skubatch wrote "DD.2 - 5'" with "PCR" directly below the DD.2 entry.

These entries reflect that she conducted another set of PCR experiments to amplify sequences corresponding to the 5' end of the DD.2 sequence. As in earlier experiments, she used different DNA libraries as a source of template DNA (e.g., Human spleen) and different PCR primers to conduct the reaction (e.g., SM.1 and DD Race 3 or DD Race 2).

334. Ms. Skubatch listed the sample numbers (1-12) along the left side of the bottom of page 93. For each library source, she used either the SM.1 and DD Race 3 primers, as in samples 1, 3, 5, 7, 9, and 11, or the SM.1 and DD Race 2 primers, as in samples 2, 4, 6, 8, 10, 12.

335. Ms. Skubatch listed the reaction conditions at the bottom right of page 93. She ran the PCR products from the samples on a gel and taped a photograph of the gel she ran at the bottom of page 93.

#### 6. Page 95, Notebook 26577 (ADE-27)

336. Ms. Skubatch conducted and recorded the activities described on page 95 on March 21, 1997, as reflected by the date recorded on that page.

337. The experiments on page 95 are a continuation of the PCR experiments described

on page 93.

338. At the top of page 95, Ms. Skubatch wrote “Transform 3 PCR products p. 93 Hu Thymus, Lung, Spleen using rapid ligations into pGEMT.” These entries reflect that she ligated three PCR products from the PCR experiments described on page 93 into a pGEMT vector using a rapid ligation kit. Based on the markings she made on the photograph of the gel on page 93, she used the PCR products from samples 2, 4, and 8, corresponding to the spleen, thymus, and lung samples, respectively.

339. Following ligation into the pGEMT vector, Ms. Skubatch transformed JM109 bacteria, incubated the cells, and grew the cells to allow for propagation of the ligated PCR products in the bacteria. She recorded the assay conditions at the middle of page 95.

**7. Pages 1 and 2 Notebook 27236 (ADE-28)**

340. Ms. Skubatch conducted and recorded the activities described on pages 1-2 on March 24, 1997, as reflected by the date recorded on those pages.

341. The experiments on page 1 are a continuation of the PCR experiments she described on pages 93 and 95 of her notebook 26577. The experiments on page 2 are a new set of experiments involving PCR of the DNA encoding a DD.2-IgG fusion construct.

342. The image below reflects entries Ms. Skubatch made at the middle of page 1.

6' of DD.2  
 Religate into pGEMT and transform  
 1 ul pGEMT  
 7 ul ~~vector~~ insert  
 10 ul vial one quick ligation kit  
 2 ul vial two " " "  
 Mix  
 1 ul vial three  
 R.T. 5'  
 add 100 ul JM109 Promega  
 20' 0° 45" 42° 2' 0°  
 add 300 ul LB spin 45' 37°  
 plate on (LB + Carb + IPTG - X-GAL) O.N. 37°

SLA - pGEM-T  
 1A Hu Thymus  
 2A Hu Lung  
 3A Hu Spleen

343. Ms. Skubatch's entries reflect that she continued with PCR experiments designated "5" of DD.2". She noted "Religate into pGEMT and transform" and listed at the right side of page 1 three samples (1A, 2A, and 3A) corresponding to the human thymus, lung, and spleen PCR products she first described on page 95 of notebook 26577. The remaining entries on the page reflect the reagents she used to ligate the PCR products into the pGEMT vector and the protocol she followed to transform "JM109" cells and plate the cells on "(LB + Carb + IPTG-X-gal)" overnight at 37°. The plating conditions reflect use of "LB" agar supplemented with carbenicillin (a selection antibiotic) and IPTG (a reagent that induces expression) and X-gal (a substrate used to detect expression of the *lacZ* marker gene which is induced by IPTG). The additional reagents added to the agar are used as confirmation that the transformation systems is functional. For example, if cells survive on carbenicillin, that indicates that the cells incorporated the pGEMT plasmid harboring the gene providing carbenicillin resistance. Similarly, if the cells stain blue, following incubation with the X-gal substrate that reflects that the *lacZ* gene encoding beta galactosidase is expressed following addition of IPTG and

functional because the enzyme converts the X-gal substrate to a blue stain.

344. Ms. Skubatch made the entries below at the top of page 2.

From Page No. \_\_\_\_\_

DD.2 - IgG Fusion


PCR

0.5 ul DD.2.1 DNA rxn 1: sm.1  
1.0 ul sm.1 or sm.2 rxn 2: sm.2  
1.0 ul sm.4  
10 ul 10x PCR Buffer  
24 ul 1.25 mM dNTP's  
1.0 ul Tag  
63 ul dH<sub>2</sub>O

94° 1' 780° 1' 72° 2' 16 cycles

Run 10 ul on 6% TBE

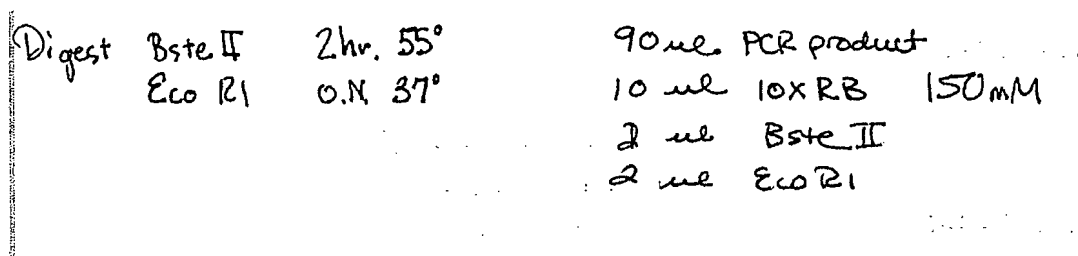
Digest Bste II 2 hr. 55° 90 ul PCR product  
Eco RI 0. N. 37° 10 ul 10x RB 150 mM  
2 ul Bste II  
2 ul Eco RI



345. The entries Ms. Skubatch made on page 2 reflect that she conducted a PCR experiment to construct a DNA encoding a DD.2-IgG fusion protein. She recorded the reaction conditions under the “PCR” entry she made at the top of page 2. Ms. Skubatch wrote “rxn 1: sm.1” and “rxn 2: sm.2” near the top of page 2 which reflects that she used either the “sm.1” PCR primer or the “sm.2” PCR primer in the experiments in conjunction with the sm.4 primer she listed under the reagents. She received the primers and the DD.2-1 DNA construct from me. The sm.1 and sm.2 primers correspond to sequence at the 5 prime end of the extracellular domain. Sm.4 corresponds to sequence on the 3 prime end of the extracellular domain close to the transmembrane domain. Ms. Skubatch recorded the PCR reaction conditions and noted “Run 10ul on 6% TBE” reflecting that she ran an aliquot of the PCR products on a gel. She taped a photograph of the gel on the top right portion of the page.

346. Following the PCR reactions, Ms. Skubatch conducted a restriction digest of the

PCR products using the restriction enzymes BstEII and EcoRI and purified the fragments, as reflected in the entries in the image of page 2 below. She listed the reagents she used in the digest on the left side of the image of page 2 below.



Handwritten notes from a notebook page, likely page 2, detailing the reagents used for a DNA digest. The notes are organized into two columns. The left column, under the heading 'Digest', lists the enzymes 'BstE II' and 'Eco RI'. The right column lists the reaction conditions and reagents: '2 hr. 55°', 'O.N. 37°', '90 ul PCR product', '10 ul 10x RB', '150 mM', '2 ul BstE II', and '2 ul Eco RI'.

Digest	Reagents
BstE II	2 hr. 55°
Eco RI	O.N. 37°
	90 ul PCR product
	10 ul 10x RB
	150 mM
	2 ul BstE II
	2 ul Eco RI

#### 8. Page 3, Notebook 27236 (ADE-28)

347. Ms. Skubatch conducted and recorded the activities described on page 3 on March 25, 1997, as reflected by the date recorded on those pages.

348. The experiments Ms. Skubatch recorded on page 3 are a continuation of the DD.2 5' ligations she described on page 1.

349. At the top of page 3, Ms. Skubatch wrote "Miniscreen via PCR DD.2 5' ligations lig #1" reflecting that she was going to screen the first ligations she made into the pGEMT vectors she described on page 1 to evaluate whether the PCR products were ligated into the pGEMT vectors. Ms. Skubatch recorded the reagents she used in the PCR reaction as well as the reaction conditions on the left side and at the top of page 3. She ran the PCR products on a gel and taped a photograph of the gel to the top of page 3. Her activities are reflected in the image of page 3 below.

Project No. \_\_\_\_\_  
Book No. 27236

TITLE \_\_\_\_\_

From Page No. \_\_\_\_\_

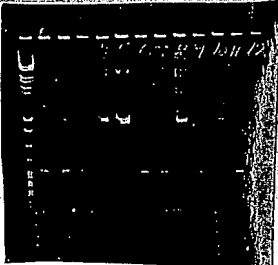
Tuesday 3/25/97

Miniscreen via PCR DD.2.5' ligations lig. #1

10 ul dH <sub>2</sub> O	15
5 ul PCR buffer	75
12 ul 1.25 mM dNTPs	180
0.5 M13f	7.5
0.5 M13r	7.5
32 ul dH <sub>2</sub> O	330
0.5 Taq	7.5

94° 1' 50° 1' 72° 1' 30 cycles

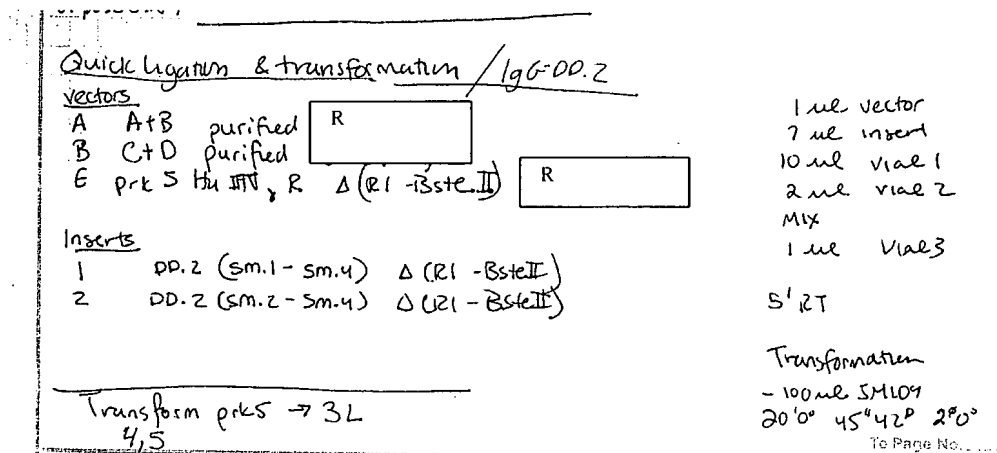
work up #4,5



350. Ms. Skubatch noted below the PCR reaction conditions “work up #4, 5” reflecting that samples in lanes labeled 4 and 5 appeared to yield the correct banding pattern and would be purified and larger quantities of the samples would be made for use in other experiments. At the bottom of page 3, she wrote “Transform prk5 → 3L” over “4,5” which reflects that Ms. Skubatch made a three liter preparation of pRK5 and the standard 500 ml preparation for clones designated #4 and #5. Though difficult to see on the image above, she made two stars in lanes labeled 4 and 5 reflecting that the samples loaded in these lanes yielded the expected banding pattern for the DD.2 PCR products.

351. At the bottom of page 3, Ms. Skubatch wrote “Quick Ligation & transformation / IgG-DD.2” reflecting that she conducted a ligation and transformation experiment using the IgG-DD.2 construct. The experiments described at the bottom of page 3 are a continuation of the experiments described on page 2. The image of page 3 below reflects these activities.





352. Under “Inserts” Ms. Skubatch wrote “DD.2 (sm.1-sm.4)  $\Delta(R1-BsteII)$ ” reflecting that the inserts she used in this ligation were from the EcoR1 and BsteII digests she conducted and described on page 2. Under “Vectors” she listed the vectors into which she ligated the DD.2 inserts. The vectors were provided to Ms. Skubatch by me.

353. At the right side of the image above, Ms. Skubatch listed the reagents she used in the ligation experiment and recorded the transformation procedure she used to transform JM109 cells with the ligated vectors (i.e., “Transformation; -100ul JM109; 20’ 0° 45’’ 42° 2’0°”).

#### 9. Pages 4-5, Notebook 27236 (ADE-28)

354. Ms. Skubatch conducted and recorded the activities described on pages 4-5 on March 26, 1997, as reflected by the dates recorded on those pages.

355. The experiments described on page 4 are a continuation of the DD.2-IgG ligations and transformations she described on page 3.

356. Ms. Skubatch conducted PCR on the ligations she made on page 3 as reflected in the image of her entries on page 4 below.

From Page No. _____			
<u>Wednesday 3/26/97</u>			
<u>PCR ligations IgG-DD.2</u>			
No.	Ligation	10 $\mu$ l dH <sub>2</sub> O	x 45 R <sub>uns</sub>
1-8	1A	12 $\mu$ l 125 mM dNTPs	540
9-21	2A	0.5 $\mu$ l sm2	22.5
22-26	B1	0.5 $\mu$ l sm4	22.5
27-30	B2	0.5 $\mu$ l Tag	22.5
31-35	E1	5 $\mu$ l PCR Buffer	22.5
36-44	E2	22 $\mu$ l dH <sub>2</sub> O	990
<u>94° 60° 72° 25 cycles</u>			

357. Along the left side of page 4, as reflected in the image above, Ms. Skubatch listed sample numbers 1-44 and the corresponding ligations (from page 3) that would be included in each sample. The ligation designations correspond to the vectors listed on page 3 (either vector A, B, or E) and one of the inserts listed on page 3 (insert 1 = DD.2 (sm.1-sm.4)  $\Delta$  (R1-BSTEII) or insert 2 = DD.2 (sm.2-sm.4)  $\Delta$  (R1-BSTEII)). Therefore, for example, ligation "1A" reflects insert number 1 ligated into vector "A", and ligation "E2" reflects insert 2 ligated into vector "E."

358. Ms. Skubatch listed the reagents she used in the PCR reaction and the reaction conditions on the right side and middle of page 4, respectively.

359. Ms. Skubatch ran the PCR products on gels and taped labeled photographs of the gels to page 4. She labeled the lanes on photos of the gels corresponding the sample numbers described above and marked lanes 10, 31, and 41. These markings reflect that the PCR products in these lanes yielded a banding pattern having the expected PCR product.

360. Ms. Skubatch noted "Submit IgG DD.2; #10, 31, and 41 for sequencing" reflecting that she would submit the DNA from samples 10, 31, and 41 to the Genentech

sequencing facility for sequencing.

361. On the top of page 5, Ms. Skubatch wrote “pick #10, 30, 31, 41” for maxi prep” reflecting that she picked DNA from samples 10, 30, 31, and 41 from the PCR experiments described on page 4 to make large scale plasmid preparations.

362. Ms. Skubatch further noted at the top of page 5, “Maxiprep 4, 5, 5xprk5” which reflects that she conducted a large scale plasmid preparation of samples 4, 5, and made five bottles of pRK5 vector. The above noted samples 4 and 5 originate from the “DD.2 5” ligations described on page 3 which Ms. Skubatch noted that she should “work up.”

**10. Page 6, Notebook 27236 (ADE-28)**

363. Ms. Skubatch conducted and recorded the activities described on page 6 on March 31, 1997, as reflected by the dates recorded on those pages.

364. The experiments described on page 6 are a continuation of the experiments Ms. Skubatch designated DD.2-5’ using PCR to amplify 5 prime sequences of the DD.2 DNA.

365. Under the “PCR” entry at the middle of page 6, Ms. Skubatch noted “DD.2-5’ = try to pull 5’ again using more external primers” which reflects that she conducted PCR experiments using different primers than previously used in similar experiments to amplify the 5 prime end of the DD.2 sequence.

366. Ms. Skubatch listed samples numbered 1-12 toward the bottom of page 6 and noted that she used a human lung library in the PCR reactions (“Hu lung”). Under the “Primers” entry she listed the primers that she used for each sample (samples 1-3 used sm.1 as one primer for the samples, and race.1, race. 2 and race.3 as the second primer for samples 1, 2, and 3, respectively; samples 4-6 used sm.2 as one primer for the samples, and race.1, race.2, and race.3 as the second primer for samples 4, 5, and 6, respectively. She noted for samples 7-12 (using the fetal lung library) that she used the same primer setup as those for samples 1-6.

367. Ms. Skubatch listed the reagents she used in the PCR experiment on the right side at the bottom of page 6 and further listed the PCR reaction conditions at the bottom left side of page 6.

368. Ms. Skubatch ran the PCR products on a 6% gel, as reflected by the entry “Run on 6% gel” and she taped a photograph of the gel at the bottom right of page 6. She marked lanes labeled 1 and 2 (corresponding to the human lung library using primers sm.1 and race.1 and sm.1 and race.2, respectively) with stars reflecting that these lanes had the expected banding pattern for the PCR experiment she conducted. Ms. Skubatch noted “Purify 1 & 2” at the very bottom of page 6, reflecting that she would purify the PCR products in samples 1 and 2.

**11. Pages 7-8, Notebook 27236 (ADE-28)**


369. Ms. Skubatch conducted and recorded the activities described on pages 7-8 on April 1, 1997, as reflected by the dates recorded on those pages.

370. The experiments described on the top half of page 7 are a continuation of the experiments she described on page 6 using the DD.2-5' sequences, whereas the experiments described on the bottom half of page 7 and the top of page 8 are a continuation of experiments described on pages 3 and 4 involving the DD.2-IgG fusion construct.

371. Ms. Skubatch wrote at the top of page 7 “5'-DD.2” over “Purify 1,2, on a column” reflecting that the experiments she described on page 7 were experiments using the 5 prime DD.2 sequences and that she purified the PCR products from samples 1 and 2 using a Promega PCR purification kit.

372. Ms. Skubatch wrote “Ligation” toward the top of page 7 reflecting that following purification of the PCR products from samples 1 and 2, she ligated the purified sample 1 and 2 inserts into a pGEMT vector. She recorded the reagents she used for the ligation at the top right side of page 7.

373. Ms. Skubatch made the following entries toward the bottom of page 7 reflecting that she continued her work on the DNA encoding the DD.2-IgG fusion protein from page 4.

DD.2-IgG			
Digest	DD.2 IgG #41	1ul DNA	
	DD.2 IgG #10	2ul 150mM 10x12B	
	DD.2 IgG #31	6.5ul BstII	
		<del>0.5ul B</del>	
Digest w/ R1 BstII		15ul dH <sub>2</sub> O	55° 30'
		1 ul R1	37° 1 hr
			
Witnessed & Understood by me.	Date	Invented by	Date
		Recorded by	

374. The entries at the bottom of page 7 in the image above reflect that Ms. Skubatch conducted a restriction digest of the DD.2 constructs designated #41, #10, and #31 (see page 4) using the restriction enzymes EcoR1 and BstII (“Digest w/ R1 BSTEII”). She recorded the reagents she used for the digests on the right side at the bottom of page 7. She ran the restriction products on a gel and taped a photograph of the gel at the bottom of page 7.

375. At the top of page 8, Ms. Skubatch made the entry provided in the image from page 8 below. The entry reflects that she repeated the DD.2-IgG ligation first described on page 3, using the vector designations “A, B, E” and the inserts designated “1,2”. She made a note to “screen uIgG.4-sm.1” which reflects that when she was ready to conduct the screen, she screened the ligation with primers corresponding to the uIgG.4 and sm.1 primers.

## 12. Page 9, Notebook 27236 (ADE-28)

376. Ms. Skubatch conducted and recorded the activities described on page 9 on April 2, 1997, as reflected by the dates recorded on those pages.

377. The experiments described on page 9 are a continuation of two experiments. Ms. Skubatch conducted PCR screens of 1) re-ligations of the DD.2-IgG fusion constructs she described on pages 3, 4, and 8; and 2) the DD.2-5' ligations she described on page 6 and the top portion of page 7.

378. An image of page 9 reflecting Ms. Skubatch's activities is set forth below.

**A** **B**

Wednesday 4.2.97

PCR Screening

#1-48 (IgG-DD.2 Samples)

1-5	1A	10 ml dH <sub>2</sub> O + plug	<del>24</del> 50	25
6-14	2A	0.5 IgG.4	<del>24</del> 25	12.5
15-25	1B	0.5 sm.1	<del>24</del> 25	12.5
26-28	2B	0.5 Tag	<del>24</del> 25	12.5
29-38	1E	5 PCR Bmgs	<del>24</del> 250	125
39-44	2E	12 dNTP's	<del>24</del> 200	300
		22 dH <sub>2</sub> O	<del>10.5</del> 1100	550

#49-72 (DD.2-5') *ligation*

#49-60 1A (M13 for M13 rev) same ↑

61-72 2A

94° 1' 60° 1' 72° 3' 35 cycles → plasmid prep of #5 "A+B / sm.1 - sm.4" digest w/ P1 - Bstc II

379. The first set of entries corresponding to arrow "A" reflects that Ms. Skubatch listed samples "#1-48" with the corresponding designations from the IgG-DD.2 ligations. The IgG-DD.2 ligations were described on pages 3, 4, and 8 and have the same "1A, 2A, 1B, 2B, 1E, 2E" designations as reflected at the top of page 9. Her entries reflect that samples 1-5 correspond to the 1A ligations; samples 6-14 correspond to the 2A ligations; samples 15-25 correspond to the 1B ligations; samples 26-28 correspond to the 2B ligations; samples 29-38 correspond to the 1E ligations; and samples 39-44 correspond to the 2E ligations. Ms. Skubatch appears to have made an error when she numbered the samples "#1-48." She should have labeled the samples 1-44 because there were only 44 samples in the IgG-DD.2 ligations described on page 4.

380. Ms. Skubatch listed the reagents (including primers) that she used in the PCR screen to the right of the IgG-DD.2 sample designations.

381. The set of entries corresponding to arrow “B” on the image above reflects that Ms. Skubatch conducted a PCR screen using the DD.2-5’ human lung ligations she described on page 6 and at the top of page 7. She labeled these samples “#49-72.” Sample numbers 49-60 correspond to the 1A ligations and samples 61-72 correspond to the 2A ligations, first described on page 3 and 4.

382. Ms. Skubatch noted that she used the same PCR screening protocol for samples 49-72 as the samples 1-48 except that she used primers designated “M13” in both the forward and reverse directions, for samples 49-72 rather than the IgG.4 and sm.1 primers she used for samples 1-48.

383. Ms. Skubatch conducted the PCR reactions and ran the PCR products on several gels and taped photographs of the gels to the bottom of page 9. She marked one image with a star at lane 5 reflecting that the band in lane 5 corresponded to PCR products having a band of the expected size for the DD.2-IgG DNA. She wrote “plasmid prep of #5 ‘A+B/sm.1-sm.4’; digest w/ R1-BstEII” reflecting that she created a plasmid preparation using the number 5 DD.2-IgG PCR product. The entry “digest w/ R1-BstEII” reflects that Ms. Skubatch was to conduct a restriction digest of the DD2-IgG plasmid using the restriction enzymes EcoR1 (“R1”) and BstEII.

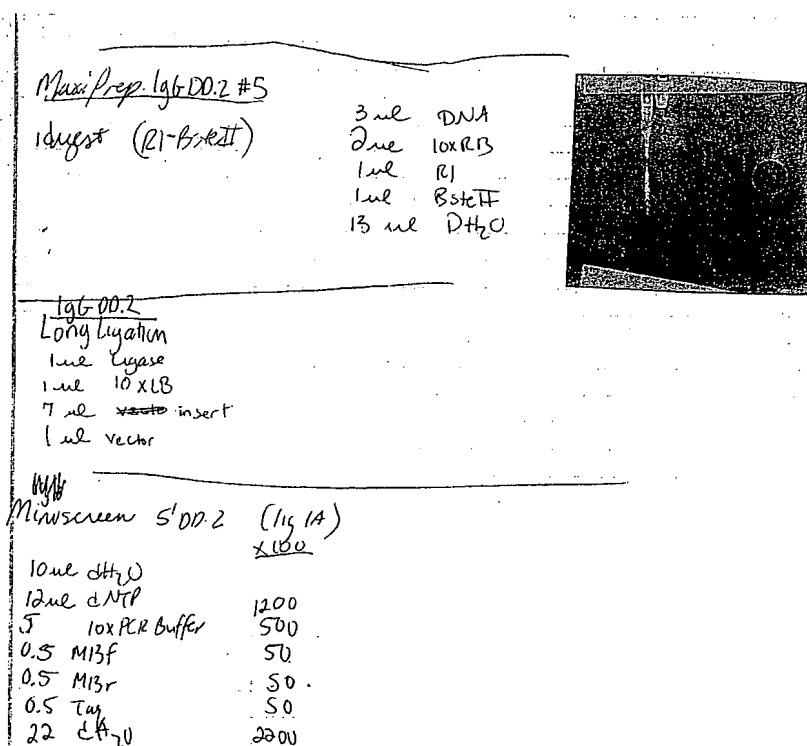
384. Ms. Skubatch wrote “Pick 96 of 1A for miniscreen” at the bottom of page 9 which reflects that, at my request, she picked 96 additional colonies that were transformed as described for the “1A” described on page 9. I asked her to pick additional colonies to continue to search for positive clones.

13. Page 11, Notebook 27236 (ADE-28)

385. Ms. Skubatch conducted and recorded the activities described on page 11 on April 3, 1997, as reflected by the date recorded on that page.

386. Ms. Skubatch conducted and recorded the activities described on page 11 on April 3, 1997, as reflected by the date she recorded on that page. The experiments described on page 11 are a continuation of two experiments. She conducted a "maxiprep" (plasmid preparation and purification) of the plasmid preparations, and then she digested the plasmid preparations using restriction enzymes. She also conducted a PCR screen of the additional ninety-six samples that I asked her to pick derived from the "1A" samples from the DD.2-5' ligation described on page 7.

387. An image of page 11 reflecting Ms. Skubatch's activities is provided below.



388. The entry "MaxiPrep IgGDD.2 #5; digest (R1-BstEII)" Ms. Skubatch made reflects that she conducted a restriction digest of previously purified PCR products from the sample #5 described on page 9. She ran the digest on a gel and taped a photograph of the gel at



the middle of page 11.

389. The “Miniscreen 5’DD.2 (lig 1A)” entry Ms. Skubatch made reflects that she conducted a PCR screen of the PCR products from the “1A” DD.2-5’ human lung ligation she described on page 7. She used M13 PCR primers in both the front and reverse direction in the screen as reflected by the “M13” entries she made in the list of reagents she used in the PCR screen at the bottom of page 11.

390. Ms. Skubatch taped a pocket to page 11 and included photographs of the gels she ran containing the PCR products from the screens she conducted. There are no markings on the images and it appears that this experiment was unsuccessful.

**14. Pages 12-13 Notebook 27236 (ADE-28)**

391. Ms. Skubatch conducted and recorded the activities described on pages 12-13 on April 4, 1997, as reflected by the dates recorded on those pages.

392. The experiments described on page 12 reflect that she conducted PCR experiments to amplify the DD.2 insert of the DD.2-IgG fusion.

393. At the top of page 12, Ms. Skubatch wrote “DD.2-IgG Construct; PCR the DD.2 Insert” reflecting that she conducted a PCR experiment to amplify the DD.2 insert in the fusion construct. Ms. Skubatch listed the reagents she used in the reaction at the top of the page which included DD.2 DNA and either sm.1 or sm.2 primers (“sm.1/sm.2”) in conjunction with the “sm.4” primer. She recorded the PCR reaction conditions and ran the PCR products on a gel. She taped a photograph of the gel to the top of page 12.

394. Following the PCR reaction, Ms. Skubatch conducted a restriction digest of the PCR products as reflected by her entry “PCR Product Digestion w/BstII and EcoR1.” She listed the reagents and reaction conditions for the digest at the middle of page 12.

395. At the top of page 13, Ms. Skubatch wrote “DD.2-5’ End; PCR HuLung”

reflecting that she continued my PCR experiments to amplify DD.2-5' sequences from a human lung library. She listed the reaction numbers 1-9 under the "Rxn#" entry on the right side of the page. To the right of each reaction number she recorded the PCR primers she used for each sample (*e.g.*, reaction 1 primers were "prk-rev" and "DD.race.1"). Ms. Skubatch listed the reagents she used and the PCR reaction conditions at the right side of page 13. She noted "Run 10ul on 6% TBE; Refrigerate" reflecting that she ran ten microliter samples on a 6% gel and refrigerated the rest of the PCR reaction.

**15. Page 16, Notebook 27236 (ADE-28)**

396. Ms. Skubatch conducted and recorded the activities described on page 16 on April 23, 1997, as reflected by the date recorded on that page.

397. At the middle of page 16, Ms. Skubatch wrote "DD.2 ECD-flag" over "Quick Transformation 1000ml→4L" reflecting that she used a construct designated DD.2-ECD-flag in a quick transformation experiment. The entry "Quick Transformation 1000ml→4L" reflects that Ms. Skubatch used a one liter volume (1000ml) of LB broth in a four liter (4L) flask to grow the bacteria which had been quick transformed with DD.2-ECD Flag.

398. Ms. Skubatch received the DD.2-ECD-Flag construct from me. The DD.2-ECD-Flag construct was a fusion construct encoding the extracellular domain ("ECD") of the DD.2 protein ligated to a "FLAG" tag. A FLAG tag is used in conjunction with a FLAG-specific antibody, or other flag-specific reagents, to purify or isolate molecules having the flag tag fused thereto.

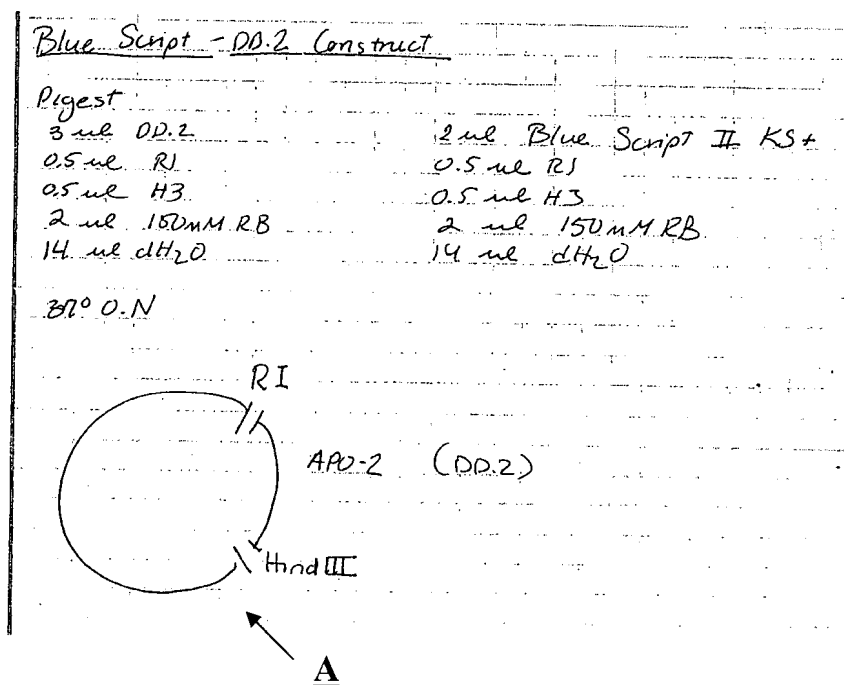
**16. Page 17, Notebook 27236 (ADE-28)**

399. Ms. Skubatch conducted and recorded the activities described on pages 17 on April 24, 1997, as reflected by the dates recorded on those pages.

400. Ms. Skubatch wrote "MaxiPrep" at the top of page 17 and listed "DD.2-ECD

FLAG” under the Maxiprep entry reflecting that she conducted a maxiprep DNA purification of DD.2-ECD FLAG DNA. The source of the DD.2-ECD-FLAG DNA was from the transformations she conducted on April 23 and described on page 16.

401. At the bottom of page Ms. Skubatch made the following entries reflecting that she made a DD.2 construct in a BlueScript plasmid. BlueScript was a commercially available vector routinely used for making DNA constructs.



402. The notation at arrow “A” shows that Ms. Skubatch drew a schematic of the BlueScript vector and included the restriction cuts that would be made following digestions with EcoR1 (“R1”) and HindIII, and the “Apo-2 (DD.2)” insert that would be ligated at the two restriction sites. She listed the digestion conditions below the “Blue Script – DD.2 Construct” entry at the middle of page 17. She allowed the digest to progress at 37 degrees Celsius overnight (“37° O.N.”).

**17. Page 18 and Page 23, Notebook 27236 (ADE-28)**

403. Ms. Skubatch conducted and recorded the activities described on page 18 on April

25, 1997, as reflected by the date she recorded on that page. Ms. Skubatch conducted and recorded the activities described on page 23 on April 28, 1997, as reflected by the date recorded on page 22. The activities she conducted on page 23 completed the experiments described on page 18.

404. At the middle of page 18, Ms. Skubatch wrote “redigest” and listed the reagents she used in the “redigest.” The reagents she listed include “3 ul DD.2” DNA and “2ul Bscript KS +” reflecting that she re-digested the BlueScript construct she first described on page 18. She noted “2hrs 37°” reflecting that she ran the digest for two hours at 37 degrees Celsius.

405. On page 23, Ms. Skubatch wrote “Run Digest from Friday” reflecting that she ran the DD.2 – BlueScript digests on a gel. According to her notebook, the only digests she ran on Friday were the DD.2 BlueScript digests. She labeled the 6<sup>th</sup> lane on photograph of the gel she taped to page 23 with “DD.2 R1-H3” reflecting the DD.2 DNA was digested with EcoR1 and HindIII restriction enzymes.

406. Ms. Skubatch noted below the gel “Cut and refrigerate” reflecting that she cut out the bands from the gel containing the DD.2 digest products and put them in a refrigerator.

**18. Page 26, Notebook 27236 (ADE-28)**

407. Ms. Skubatch conducted and recorded the activities described on page 26 on April 29, 1997, as reflected by the date she recorded on page 24. Her activities relevant to the Apo-2 project are described on page 26.

408. At the top of page 26 Ms. Skubatch wrote “...Apo-2-BlueScript” reflecting that she continued work on making an Apo-2 BlueScript construct. She also wrote “Quick Ligation Kit” at the top of page 26 reflecting that she used a quick ligation kit to ligate the DD.2 insert into the BlueScript vector.

409. Ms. Skubatch made the entries in the image below at the middle of page 26

reflecting the reagents she used in the ligation experiment.

SLB 1 ul BS +      1 ul BS  
 7 ul H<sub>2</sub>O      7 ul DD.2  
 1 ul LB vial 1      1 ul LB vial 1  
 1 ul ligase      1 ul ligase  
 2 ul vial 2      2 ul vial 2

RT 10'

---

Transform 100 ul JM109      20' @ 45° 42', 2' @ 45° 37' on wheel

plate      Run fragments on gel

410. The “BS” entries in the reagents list reflects that Ms. Skubatch used the BlueScript vector, and the “DD.2” entry reflects that she used DD.2 DNA as an insert to ligate into the BlueScript vector. Following ligation, she noted “Transform 100ul JM109” reflecting that she transformed JM109 bacterial cells with the ligated vectors. She listed the transformation conditions immediately to the right of the “JM109” entry. She then plated the transformed bacteria to allow bacterial growth and propagation of the BlueScript vector carrying the DD.2 insert.

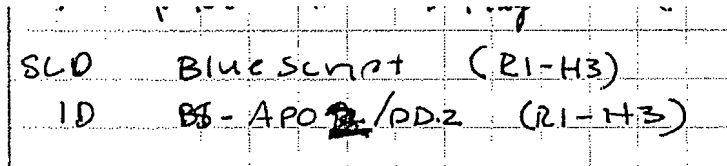
**19. Page 29, Notebook 27236 (ADE-28)**

411. Ms. Skubatch conducted and recorded the activities described on page 29 on April 30, 1997, as reflected by the date she recorded on page 28. Her activities relevant to the Apo-2 project are described on page 29.

412. Ms. Skubatch noted at the top of page 29 “Long Ligations” and made several entries describing the ligations that she conducted. She made the entry before the line across the middle of the page which reads “1D BS-Apo-2/DD.2 (R1-H3).” These entries reflect that she conducted a long ligation experiment using the BlueScript vector and the Apo-2/DD.2 insert

previously digested with EcoR1 (R1) and HindIII (H3). She used the Apo-2 and DD.2 designations interchangeably because by this time the DD.2 DNA was being referred to as the Apo-2 receptor.

413. The image below from page 29 reflects these activities.



**20. Pages 32-33, Notebook 27236 (ADE-28)**

414. Ms. Skubatch conducted and recorded the activities described on pages 32 and 33 on May 2, 1997, as reflected by the date she recorded on page 32.

415. Ms. Skubatch wrote at the top of page 32 “PCR ligations” reflecting that she conducted PCR’s of ligations from page 29.

416. Ms. Skubatch listed several sample numbers along the left side of the page including sample number 74 designated “DD.2.” She noted that she used the “prkfor” and “prkrev” PCR primers for experiments using DD.2. The “prk” primers were complementary to sequences contained in the prk5 vector in which the DD.2 sequence was initially cloned.

417. Ms. Skubatch listed the PCR reagents and reaction conditions at the top right side of page 32 and ran the samples on gels.

418. Ms. Skubatch noted that “Nothing Came out on the Gels” reflecting that these experiments were unsuccessful.

419. At the top of page 33, Ms. Skubatch wrote “Apo-2/SCRIP” reflecting that the experiments described on page 33 involved trying to make an Apo-2 –BlueScript construct. She wrote “Digest” reflecting that the experiment she conducted was a digest of “DD.2/Apo-2” DNA for ligation into BlueScript vector. She listed the reagents used in the digest including the

restriction enzymes PVUI, EcoRI, and HindIII.

420. Ms. Skubatch ran the digest for two hours at 37 degrees Celsius ("2h 37°"). She recorded reaction conditions she followed after the digest described at the middle of page 33.

**21. Page 35, Notebook 27236 (ADE-28)**

421. Ms. Skubatch conducted and recorded the activities described on page 35 on May 5, 1997, as reflected by the date she recorded on page 35.

422. Ms. Skubatch made the following entries at the bottom of page 35 reflecting that she conducted a ligation of Apo-2 DNA into a BlueScript vector:

Ligation / Quick Ligation of BlueScript Vector + Apo2	
1 ml Blue Script	1 ml Blue Script
7 ml Apo2 / dH <sub>2</sub> O	7 ml Apo2 / dH <sub>2</sub> O
10 ml vial 1 (CAL)	1 ml 10x LB (L.L)
2 ml vial 2 (CAL)	1 ml 10mM ATP (L.L)
1 ml vial 3 (CAL)	1 ml vial 5
O.N. 14°	5' R.T.
	Transform, use 100 µl JM109 20'0°, 45'42°, 2'00
	Plate on LB + Carb

423. The column on the left reflects the reagents Ms. Skubatch used in conducting a ligation of Apo-2 DNA into the "Blue Script" vector. She incubated the ligation overnight at 14 degrees Celsius as reflected by the "O.N. 14°" entry.

424. The column on the right reflects the reagents Ms. Skubatch used in conducting a "Quick Ligation" of Apo-2 DNA into the "Blue Script" vector. She incubated the reaction for five minutes at room temperature ("5' R.T.") and transformed the ligated vector into JM109 bacterial cells ("Transform use 100ul JM109"). She recorded the transformation conditions to the right of the "JM109" entry and noted that she plated the transformed cells on LB agar broth supplemented with the antibiotic carbenicillin to select for transformants as reflected in the entry

“LB + Carb.”

**IV. Conclusion**

425. I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of any patents issuing from the above-identified application.

Date: 4/20/07

  
Scot Marsters



>From aa [REDACTED]

Date: [REDACTED]

X-Sender: aa@ruby.gene.com

Mime-Version: 1.0

To: wiw@gene.COM

From: aa@gene.COM (Avi Ashkenazi)

Subject: incyte sequences

So far there are 3 sets of Incyte sequences that we plan to clone out:

1. 1353959, 1353903, (cluster 78530), homologous to Apo-2L
2. Cluster 16411 (10 sequences), homologous to Apo-2L
3. Cluster 75799 (2 sequences), homologous to the Apo-3 death domain.

**ADE- 7**

USSN 10/052,798

>From aa [REDACTED]  
>Date: [REDACTED]  
>X-Sender: aa@ruby.gene.com  
>Mime-Version: 1.0  
>To: wiw@gene.COM (William I. Wood)  
>From: aa@gene.COM (Avi Ashkenazi)  
>Subject: Re: incyte sequences

Cluster 16411: clones 1721344, 1215220, 2230166, 1802487, 1873725, 1876214, 1985662, 690050, 687247, 687534.

Cluster 75799: clones 2078364, 1237537.

>Yes, the cluster numbers ought to make sense, but they are not constant and  
>get reset with each release of the database. So I am going to need clone  
>numbers for the second two entries.

>William

>

>>So far there are 3 sets of Incyte sequences that we plan to clone out:

>>1. 1353959, 1353903, (cluster 78530), homologous to Apo-2L

>>2. Cluster 16411 (10 sequences), homologous to Apo-2L

>>3. Cluster 75799 (2 sequences), homologous to the Apo-3 death domain.

**ADE- 8**  
USSN 10/052,798

```

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/home/ruby/va/Molbio/wiw/incyte/gene6
leu> ls -l
total 24
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-rwxr-x--- 1 wiw Molbio 307 [REDACTED] ss.INC2078364*
-rwxr-x--- 1 wiw Molbio 342 [REDACTED] ss.gene6.consensus*
leu> more ss.*
::::::::::::
ss.INC1237537
::::::::::::
>1237537 LUNGTUT02 INCYTE
CTTTGACTNCTGGGAGCCGCTCATGAGGAAGTTGGGCCTCATGGACAATGAGATAAAGGT
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CAACAAAACCGGGCGAGATGCCTCTGTCCACACCCTGCTGGATGCCTTGGAGACGCTGGG
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TCTNGAAGGTAATGCAGACT
::::::::::::
ss.INC2078364
::::::::::::
>2078364 ISLTNOT01 INCYTE
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TGCTGATAAAGTGGGTCAACAAAACCGGGCGAGATGCCTCTGTCCAAACCCTGCTGGATG
CCTTGGAGACGCTGGGAGAGAGACTTGCCA
::::::::::::
ss.gene6.consensus
::::::::::::
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CCTTGGAGACGCTGGGAGAGAGACTTGCCAAGCAGANGATTGNGGACCACTTGTTGNGCT
CTGGAAAGTTCATGTATCTNGAAGGTAATGCAGACT
leu>

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PATENT DOCKET NO. P1101  
EXPRESS MAIL NO: \_\_\_\_\_  
MAILED: \_\_\_\_\_

Draft #1 - May 5, 1997

Apo-2 Receptor

FIELD OF THE INVENTION

The present invention relates generally to the identification, isolation, and recombinant production of novel polypeptides, designated herein as "Apo-2".

BACKGROUND OF THE INVENTION

Apoptosis or "Programmed Cell Death"

Control of cell numbers in mammals is believed to be determined, in part, by a balance between cell proliferation and cell death. One form of cell death, sometimes referred to as necrotic cell death, is typically characterized as a pathologic form of cell death resulting from some trauma or cellular injury. In contrast, there is another, "physiologic" form of cell death which usually proceeds in an orderly or controlled manner. This orderly or controlled form of cell death is often referred to as "apoptosis" [see, e.g., Barr et al., Bio/Technology, 12:487-493 (1994); Steller et al., Science, 267:1445-1449 (1995)]. Apoptotic cell death naturally occurs in many physiological processes, including embryonic development and clonal selection in the immune system [Itoh et al., Cell, 66:233-243 (1991)]. Decreased levels of apoptotic cell death have been associated with a variety of pathological conditions, including cancer, lupus, and herpes virus infection [Thompson, Science, 267:1456-1462 (1995)]. Increased levels of apoptotic cell death may be associated with a variety of other pathological conditions, including AIDS, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, multiple sclerosis, retinitis pigmentosa, cerebellar degeneration, aplastic

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anemia, myocardial infarction, stroke, reperfusion injury, and toxin-induced liver disease [see, Thompson, supra].

Apoptotic cell death is typically accompanied by one or more characteristic morphological and biochemical changes in cells, such as condensation of cytoplasm, loss of plasma membrane microvilli, segmentation of the nucleus, degradation of chromosomal DNA or loss of mitochondrial function. A variety of extrinsic and intrinsic signals are believed to trigger or induce such morphological and biochemical cellular changes [Raff, Nature, 356:397-400 (1992); Steller, supra; Sachs et al., Blood, 82:15 (1993)]. For instance, they can be triggered by hormonal stimuli, such as glucocorticoid hormones for immature thymocytes, as well as withdrawal of certain growth factors [Watanabe-Fukunaga et al., Nature, 356:314-317 (1992)]. Also, some identified oncogenes such as *myc*, *rel*, and *E1A*, and tumor suppressors, like *p53*, have been reported to have a role in inducing apoptosis. Certain chemotherapy drugs and some forms of radiation have likewise been observed to have apoptosis-inducing activity [Thompson, supra].

#### TNF Family of Cytokines

Various molecules, such as tumor necrosis factor- $\alpha$  ("TNF- $\alpha$ "), tumor necrosis factor- $\beta$  ("TNF- $\beta$ " or "lymphotoxin"), CD30 ligand, CD27 ligand, CD40 ligand, OX-40 ligand, 4-1BB ligand, Apo-1 ligand (also referred to as Fas ligand or CD95 ligand), and Apo-2 ligand (also referred to as TRAIL) have been identified as members of the tumor necrosis factor ("TNF") family of cytokines [See, e.g., Gruss and Dower, Blood, 85:3378-3404 (1995); Wiley et al., Immunity, 3:673-682 (1995); Pitti et al., J. Biol. Chem., 271:12687-12690 (1996)]. Among these molecules, TNF- $\alpha$ , TNF- $\beta$ , CD30 ligand, 4-1BB ligand, Apo-1 ligand, and Apo-2 ligand (TRAIL) have been reported to be involved in apoptotic cell death. Both TNF- $\alpha$  and TNF- $\beta$  have been reported to induce apoptotic death in susceptible tumor cells [Schmid et al., Proc. Natl. Acad. Sci., 83:1881 (1986); Dealtry et al., Eur. J. Immunol., 17:689 (1987)].

Zheng et al. have reported that TNF- $\alpha$  is involved in post-stimulation apoptosis of CD8-positive T cells [Zheng et al., Nature, 377:348-351 (1995)]. Other investigators have reported

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that CD30 ligand may be involved in deletion of self-reactive T cells in the thymus [Amakawa et al., Cold Spring Harbor Laboratory Symposium on Programmed Cell Death, Abstr. No. 10, (1995)].

5 Mutations in the mouse Fas/Apo-1 receptor or ligand genes (called *lpr* and *gld*, respectively) have been associated with some autoimmune disorders, indicating that Apo-1 ligand may play a role in regulating the clonal deletion of self-reactive lymphocytes in the periphery [Krammer et al., Curr. Op. Immunol., 6:279-289 (1994); Nagata et al., Science, 267:1449-1456 (1995)]. Apo-1  
10 ligand is also reported to induce post-stimulation apoptosis in CD4-positive T lymphocytes and in B lymphocytes, and may be involved in the elimination of activated lymphocytes when their function is no longer needed [Krammer et al., supra; Nagata et al., supra]. Agonist mouse monoclonal antibodies specifically binding  
15 to the Apo-1 receptor have been reported to exhibit cell killing activity that is comparable to or similar to that of TNF- $\alpha$  [Yonehara et al., J. Exp. Med., 169:1747-1756 (1989)].

#### TNF Family of Receptors

20 Induction of various cellular responses mediated by such TNF family cytokines is believed to be initiated by their binding to specific cell receptors. Two distinct TNF receptors of approximately 55-kDa (TNFR1) and 75-kDa (TNFR2) have been identified [Hohman et al., J. Biol. Chem., 264:14927-14934 (1989); Brockhaus et al., Proc. Natl. Acad. Sci., 87:3127-3131 (1990); EP  
25 417,563, published March 20, 1991] and human and mouse cDNAs corresponding to both receptor types have been isolated and characterized [Loetscher et al., Cell, 61:351 (1990); Schall et al., Cell, 61:361 (1990); Smith et al., Science, 248:1019-1023 (1990); Lewis et al., Proc. Natl. Acad. Sci., 88:2830-2834 (1991);  
30 Goodwin et al., Mol. Cell. Biol., 11:3020-3026 (1991)]. Extensive polymorphisms have been associated with both TNF receptor genes [see, e.g., Takao et al., Immunogenetics, 37:199-203 (1993)]. Both TNFRs share the typical structure of cell surface receptors including extracellular, transmembrane and intracellular regions.  
35 The extracellular portions of both receptors are found naturally

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also as soluble TNF-binding proteins [Nophar, Y. et al., EMBO J.,  
9:3269 (1990); and Kohno, T. et al., Proc. Natl. Acad. Sci. U.S.A.,  
87:8331 (1990)]. More recently, the cloning of recombinant soluble  
TNF receptors was reported by Hale et al. [J. Cell. Biochem.  
5 Supplement 15F, 1991, p. 113 (P424)].

The extracellular portion of type 1 and type 2 TNFRs  
(TNFR1 and TNFR2) contains a repetitive amino acid sequence pattern  
of four cysteine-rich domains (CRDs) designated 1 through 4,  
starting from the NH<sub>2</sub>-terminus. Each CRD is about 40 amino acids  
10 long and contains 4 to 6 cysteine residues at positions which are  
well conserved [Schall et al., supra; Loetscher et al., supra;  
Smith et al., supra; Nophar et al., supra; Kohno et al., supra].  
In TNFR1, the approximate boundaries of the four CRDs are as  
follows: CRD1- amino acids 14 to about 53; CRD2- amino acids from  
15 about 54 to about 97; CRD3- amino acids from about 98 to about 138;  
CRD4- amino acids from about 139 to about 167. In TNFR2, CRD1  
includes amino acids 17 to about 54; CRD2- amino acids from about  
55 to about 97; CRD3- amino acids from about 98 to about 140; and  
CRD4- amino acids from about 141 to about 179 [Banner et al., Cell,  
20 73:431-435 (1993)]. The potential role of the CRDs in ligand  
binding is also described by Banner et al., supra.

A similar repetitive pattern of CRDs exists in several  
other cell-surface proteins, including the p75 nerve growth factor  
receptor (NGFR) [Johnson et al., Cell, 47:545 (1986); Radeke et  
25 al., Nature, 325:593 (1987)], the B cell antigen CD40 [Stamenkovic  
et al., EMBO J., 8:1403 (1989)], the T cell antigen OX40 [Mallet et  
al., EMBO J., 9:1063 (1990)] and the Fas antigen [Yonehara et al.,  
supra and Itoh et al., supra]. CRDs are also found in the soluble  
TNFR (sTNFR)-like T2 proteins of the Shope and myxoma poxviruses  
30 [Upton et al., Virology, 160:20-29 (1987); Smith et al., Biochem.  
Biophys. Res. Commun., 176:335 (1991); Upton et al., Virology,  
184:370 (1991)]. Optimal alignment of these sequences indicates  
that the positions of the cysteine residues are well conserved.  
These receptors are sometimes collectively referred to as members  
35 of the TNF/NGF receptor superfamily. Recent studies on p75NGFR

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showed that the deletion of CRD1 [Welcher, A.A. et al., Proc. Natl. Acad. Sci. USA, 88:159-163 (1991)] or a 5-amino acid insertion in this domain [Yan, H. and Chao, M.V., J. Biol. Chem., 266:12099-12104 (1991)] had little or no effect on NGF binding [Yan, H. and Chao, M.V., supra]. p75 NGFR contains a proline-rich stretch of about 60 amino acids, between its CRD4 and transmembrane region, which is not involved in NGF binding [Peetre, C. et al., Eur. J. Hematol., 41:414-419 (1988); Seckinger, P. et al., J. Biol. Chem., 264:11966-11973 (1989); Yan, H. and Chao, M.V., supra]. A similar proline-rich region is found in TNFR2 but not in TNFR1.

Itoh et al. disclose that the Apo-1 receptor can signal an apoptotic cell death similar to that signaled by the 55-kDa TNFR1 [Itoh et al., supra]. Expression of the Apo-1 antigen has also been reported to be down-regulated along with that of TNFR1 when cells are treated with either TNF- $\alpha$  or anti-Apo-1 mouse monoclonal antibody [Krammer et al., supra; Nagata et al., supra]. Accordingly, some investigators have hypothesized that cell lines that co-express both Apo-1 and TNFR1 receptors may mediate cell killing through common signaling pathways [Id.].

The TNF family ligands identified to date, with the exception of lymphotoxin- $\alpha$ , are type II transmembrane proteins, whose C-terminus is extracellular. In contrast, the receptors in the TNF receptor (TNFR) family identified to date are type I transmembrane proteins. In both the TNF ligand and receptor families, however, homology identified between family members has been found mainly in the extracellular domain ("ECD"). Several of the TNF family cytokines, including TNF- $\alpha$ , Apo-1 ligand and CD40 ligand, are cleaved proteolytically at the cell surface; the resulting protein in each case typically forms a homotrimeric molecule that functions as a soluble cytokine. TNF receptor family proteins are also usually cleaved proteolytically to release soluble receptor ECDs that can function as inhibitors of the cognate cytokines.

Recently, other members of the TNFR family have been identified. In Marsters et al., Curr. Biol., 6:750 (1996),

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investigators describe a full length native sequence human polypeptide, called Apo-3, which exhibits similarity to the TNFR family in its extracellular cysteine-rich repeats and resembles TNFR1 and CD95 in that it contains a cytoplasmic death domain sequence [see also Marsters et al., Curr. Biol., 6:1669 (1996)]. Apo-3 has also been referred to by other investigators as DR3, wsl-1 and TRAMP [Chinnaiyan et al., Science, 274:990 (1996); Kitson et al., Nature, 384:372 (1996); Bodmer et al., Immunity, 6:79 (1997)].

Pan et al. have disclosed another TNF receptor family member referred to as "DR4" [Pan et al., Science, 276:111-113 (1997)]. The DR4 was reported to contain a cytoplasmic death domain capable of engaging the cell suicide apparatus. Pan et al. disclose that DR4 is believed to be a receptor for the ligand known as Apo-2 ligand or TRAIL.

#### The Apoptosis-Inducing Signaling Complex

As presently understood, the cell death program contains at least three important elements - activators, inhibitors, and effectors; in *C. elegans*, these elements are encoded respectively by three genes, *Ced-4*, *Ced-9* and *Ced-3* [Horvitz \_\_\_\_\_; Chinnaiyan et al., Science, 275:1122-1126 (1997)]. Two of the TNFR family members, TNFR1 and Fas/Apo1 (CD95), can activate apoptotic cell death [Chinnaiyan and Dixit, Current Biology, 6:555-562 (1996); Fraser and Evan, Cell, 85:781-784 (1996)]. TNFR1 is also known to mediate activation of the transcription factor, NF- $\kappa$ B [Tartaglia et al., Cell, 74:845-853 (1993); Hsu et al., Cell, 84:299-308 (1996)]. In addition to some ECD homology, these two receptors share homology in their intracellular domain (ICD) in an oligomerization interface known as the death domain [Tartaglia et al., supra; Nagata, Cell, 88:355 (1997)]. Death domains are also found in several metazoan proteins that regulate apoptosis, namely, the *Drosophila* protein, Reaper, and the mammalian proteins referred to as FADD/MORT1, TRADD, and RIP [Cleaveland and Ihle, Cell, 81:479-482 (1995)]. Using the yeast-two hybrid system, Raven et al. report the identification of protein, wsl-1, which binds to the TNFR1 death domain [Raven et al., Programmed Cell Death Meeting,

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September 20-24, 1995, Abstract at page 127; Raven et al., European Cytokine Network, 7:Abstr. 82 at page 210 (April-June 1996)]. The wsl-1 protein is described as being homologous to TNFR1 (48% identity) and having a restricted tissue distribution. According to Raven et al., the tissue distribution of wsl-1 is significantly different from the TNFR1 binding protein, TRADD.

Upon ligand binding and receptor clustering, TNFR1 and CD95 are believed to recruit FADD into a death-inducing signalling complex. CD95 purportedly binds FADD directly, while TNFR1 binds FADD indirectly via TRADD [Chinnaiyan et al., Cell, 81:505-512 (1995); Boldin et al., J. Biol. Chem., 270:387-391 (1995); Hsu et al., supra; Chinnaiyan et al., J. Biol. Chem., 271:4961-4965 (1996)]. It has been reported that FADD serves as an adaptor protein which recruits the Ced-3-related protease, MACH $\alpha$ /FLICE (caspase 8), into the death signalling complex [Boldin et al., Cell, 85:803-815 (1996); Muzio et al., Cell, 85:817-827 (1996)]. MACH $\alpha$ /FLICE appears to be the trigger that sets off a cascade of apoptotic proteases, including the interleukin-1 $\beta$  converting enzyme (ICE) and CPP32/Yama, which may execute some critical aspects of the cell death programme [Fraser and Evan, supra].

It was recently disclosed that programmed cell death involves the activity of members of a family of cysteine proteases related to the *C. elegans* cell death gene, *ced-3*, and to the mammalian IL-1-converting enzyme, ICE. The activity of the ICE and CPP32/Yama proteases can be inhibited by the product of the cowpox virus gene, *crmA* [Ray et al., Cell, 69:597-604 (1992); Tewari et al., Cell, 81:801-809 (1995)]. Recent studies show that CrmA can inhibit TNFR1- and CD95-induced cell death [Enari et al., Nature, 375:78-81 (1995); Tewari et al., J. Biol. Chem., 270:3255-3260 (1995)].

As reviewed recently by Tewari et al., TNFR1, TNFR2 and CD40 modulate the expression of proinflammatory and costimulatory cytokines, cytokine receptors, and cell adhesion molecules through activation of the transcription factor, NF- $\kappa$ B [Tewari et al., Curr. Op. Genet. Develop., 6:39-44 (1996)]. NF- $\kappa$ B is the prototype of a

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family of dimeric transcription factors whose subunits contain conserved Rel regions [Verma et al., Genes Develop., 9:2723-2735 (1996); Baldwin, Ann. Rev. Immunol., 14:649-681 (1996)]. In its latent form, NF- $\kappa$ B is complexed with members of the I $\kappa$ B inhibitor family; upon inactivation of the I $\kappa$ B in response to certain stimuli, released NF- $\kappa$ B translocates to the nucleus where it binds to specific DNA sequences and activates gene transcription. TNFR proteins may also regulate the AP-1 transcription factor family [Karin, J. Biol. Chem., 270:16483-16486 (1995)]. AP-1 represents a separate family of dimeric transcriptional activators composed of members of the Fos and Jun protein families [Karin, supra]. AP-1 activation is believed to be mediated by immediate-early induction of *fos* and *jun* through the mitogen-activated protein kinases ERK and JNK, as well as by JNK-dependent phosphorylation of Jun proteins [Karin, supra]. Transcriptional regulation by TNFR family members is mediated primarily by members of the TNF receptor associated factor (TRAF) family [Rothe et al., Cell, 78:681-692 (1994); Hsu et al., Cell, 84:299-308 (1996); Liu et al., Cell, 87:565-576 (1996)].

For a review of the TNF family of cytokines and their receptors, see Gruss and Dower, supra.

#### SUMMARY OF THE INVENTION

Applicants have identified cDNA clones that encode novel polypeptides, designated in the present application as "Apo-2." It is believed that Apo-2 is a member of the TNFR family; full-length native sequence human Apo-2 polypeptide exhibits some similarities to some known TNFRs, including a cytoplasmic death domain region. Full-length native sequence human Apo-2 also exhibits similarity to the TNFR family in its extracellular cysteine-rich repeats. Apo-2 polypeptide has surprisingly been found to be capable of triggering caspase-dependent apoptosis and activating NF- $\kappa$ B. Applicants found that the soluble extracellular domain of Apo-2 binds Apo-2 ligand (Apo-2L) and can inhibit Apo-2 ligand function. It is presently believed that Apo-2 ligand can signal via at least two different

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receptors, DR4 and the newly described Apo-2 herein.

In one embodiment, the invention provides isolated Apo-2 polypeptide. In particular, the invention provides isolated native sequence Apo-2 polypeptide, which in one embodiment, includes an amino acid sequence comprising residues 1 to 411 of Figure 1 (SEQ ID NO:1). In other embodiments, the isolated Apo-2 polypeptide comprises at least about 80% amino acid sequence identity with native sequence Apo-2 polypeptide comprising residues 1 to 411 of Figure 1 (SEQ ID NO:1).

In another embodiment, the invention provides an isolated extracellular domain (ECD) sequence of Apo-2. The isolated extracellular domain sequence preferably comprises residues 49 to 182 of Fig. 1 (SEQ ID NO:1).

In another embodiment, the invention provides an isolated death domain sequence of Apo-2. The isolated death domain sequence preferably comprises residues 324 to 391 of Fig. 1 (SEQ ID NO:1).

In another embodiment, the invention provides chimeric molecules comprising Apo-2 polypeptide fused to a heterologous polypeptide or amino acid sequence. An example of such a chimeric molecule comprises an Apo-2 fused to an immunoglobulin sequence. Another example comprises an extracellular domain sequence of Apo-2 fused to a heterologous polypeptide or amino acid sequence, such as an immunoglobulin sequence.

In another embodiment, the invention provides an isolated nucleic acid molecule encoding Apo-2 polypeptide. In one aspect, the nucleic acid molecule is RNA or DNA that encodes an Apo-2 polypeptide or a particular domain of Apo-2, or is complementary to such encoding nucleic acid sequence, and remains stably bound to it under at least moderate, and optionally, under high stringency conditions. In one embodiment, the nucleic acid sequence is selected from:

(a) the coding region of the nucleic acid sequence of Figure 1 (SEQ ID NO:\_\_\_) that codes for residue 1 to residue 411 (i.e., nucleotides 140-142 through 1370-1372), inclusive; or

(b) the coding region of the nucleic acid sequence of

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Figure 1 (SEQ ID NO:\_\_) that codes for residue 49 to residue 182 (i.e., nucleotides 284-286 through 673-675), inclusive;

(c) the coding region of the nucleic acid sequence of Figure 1 (SEQ ID NO:\_\_) that codes for residue 324 to residue 391 (i.e., nucleotides 1109-1111 through 1310-1312), inclusive; or

(d) a sequence corresponding to the sequence of (a), (b) or (c) within the scope of degeneracy of the genetic code.

In a further embodiment, the invention provides a vector comprising the nucleic acid molecule encoding the Apo-2 polypeptide or particular domain of Apo-2. A host cell comprising the vector or the nucleic acid molecule is also provided. A method of producing Apo-2 is further provided.

In another embodiment, the invention provides an antibody which specifically binds to Apo-2. The antibody may be an agonistic, antagonistic or neutralizing antibody.

In another embodiment, the invention provides non-human, transgenic or knock-out animals.

A further embodiment of the invention provides articles of manufacture and kits that include Apo-2 or Apo-2 antibodies.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the nucleotide sequence of a native sequence human Apo-2 cDNA and its derived amino acid sequence.

Figure 2A shows the derived amino acid sequence of a native sequence human Apo-2 - the putative signal sequence is underlined, the putative transmembrane domain is boxed, and the putative death domain sequence is dash underlined. The cysteines of the two cysteine-rich domains are individually underlined.

Figure 2B shows an alignment and comparison of the death domain sequences of native sequence human Apo-2, DR4, Apo-3/DR3, TNFR1, and Fas/Apo-2 (CD95). Asterisks indicate residues that are essential for death signaling by TNFR1 [Tartaglia et al., supra].

Figure 3 shows the interaction of the Apo-2 ECD with Apo-2L. Supernatants from mock-transfected 293 cells or from 293 cells

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transfected with Apo-2 ECD-Flag were incubated with poly-His-tagged Apo-2L and subjected to immunoprecipitation with anti-Flag conjugated or Nickel conjugated agarose beads. The precipitated proteins were resolved by electrophoresis on polyacrylamide gels, and detected by immunoblot with anti-Apo-2L or anti-Flag antibody.

Figure 4 shows the induction of apoptosis by Apo-2 and inhibition of Apo-2L activity by soluble Apo-2 ECD. Human 293 cells (A, B) or HeLa cells (C) were transfected by pRK5 vector or by pRK5-based plasmids encoding Apo-2 and/or CrmA. Apoptosis was assessed by morphology (A), DNA fragmentation (B), or by FACS (C). Soluble Apo-2L was pre-incubated with buffer or affinity-purified Apo-2 ECD together with anti-Flag antibody and added to HeLa cells. The cells were later analyzed for apoptosis by FACS (D). Dose-response analysis using Apo-2 ECD immunoadhesin was also determined (E).

Figure 5 shows activation of NF- $\kappa$ B by Apo-2, DR4, and Apo-2L. (A) HeLa cells were transfected with expression plasmids encoding the indicated proteins. Nuclear extracts were prepared and analyzed by an electrophoretic mobility shift assay. (B) HeLa cells or MCF7 cells were treated with buffer, Apo-2L or TNF-alpha and assayed for NF- $\kappa$ B activity. (C) HeLa cells were preincubated with buffer, ALLN or cyclohexamide before addition of Apo-2L. Apoptosis was later analyzed by FACS.

Figure 6 shows expression of Apo-2 mRNA in human tissues as analyzed by Northern hybridization of human tissue poly A RNA blots.

## DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

### I. Definitions

The terms "Apo-2 polypeptide" and "Apo-2" when used herein encompass native sequence Apo-2 and Apo-2 variants (which are further defined herein). These terms encompass Apo-2 from a variety of mammals, including humans. The Apo-2 may be isolated

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from a variety of sources, such as from human tissue types or from another source, or prepared by recombinant or synthetic methods.

5 A "native sequence Apo-2" comprises a polypeptide having the same amino acid sequence as an Apo-2 derived from nature. Thus, a native sequence Apo-2 can have the amino acid sequence of naturally-occurring Apo-2 from any mammal. Such native sequence Apo-2 can be isolated from nature or can be produced by recombinant or synthetic means. The term "native sequence Apo-2" specifically encompasses naturally-occurring truncated or secreted forms of the Apo-2 (e.g., an extracellular domain sequence), naturally-occurring variant forms (e.g., alternatively spliced forms) and naturally-occurring allelic variants of the Apo-2. A naturally-occurring variant form of the Apo-2 includes an Apo-2 having an amino acid substitution at residue 410 in the amino acid sequence shown in Figure 1 (SEQ ID NO:\_\_\_). In one embodiment of such naturally-occurring variant form, the leucine residue at position 410 is substituted by a methionine residue. In one embodiment of the invention, the native sequence Apo-2 is a mature or full-length native sequence Apo-2 comprising amino acids 1 to 411 of Fig. 1 (SEQ ID NO:1).

20 The "Apo-2 extracellular domain" or "Apo-2 ECD" refers to a form of Apo-2 which is essentially free of the transmembrane and cytoplasmic domains of Apo-2. Ordinarily, Apo-2 ECD will have less than 1% of such transmembrane and/or cytoplasmic domains and preferably, will have less than 0.5% of such domains. Preferably, Apo-2 ECD will comprise amino acid residues 49 to 182 of Fig.1 (SEQ ID NO:\_\_\_). **need to reconcile these numbers with the ECD referred to in Ex. 2, e.g. 1-184**

30 "Apo-2 variant" means a biologically active Apo-2 as defined below having at least about 80% amino acid sequence identity with the Apo-2 having the deduced amino acid sequence shown in Fig. 1 (SEQ ID NO:\_\_\_) for a full-length native sequence human Apo-2. Such Apo-2 variants include, for instance, Apo-2 polypeptides wherein one or more amino acid residues are added at the N- or C-terminus of the sequence of Fig. 1 (SEQ ID NO:\_\_\_).

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Ordinarily, an Apo-2 variant will have at least about 80% amino acid sequence identity, more preferably at least about 90% amino acid sequence identity, and even more preferably at least about 95% amino acid sequence identity with the amino acid sequence of Fig. 1 (SEQ ID NO: \_\_\_\_).

"Percent (%) amino acid sequence identity" with respect to the Apo-2 sequences identified herein is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the Apo-2 sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as \_\_\_\_\_. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared.

The term "epitope tagged" when used herein refers to a chimeric polypeptide comprising Apo-2, or a domain sequence thereof, fused to a "tag polypeptide". The tag polypeptide has enough residues to provide an epitope against which an antibody can be made, yet is short enough such that it does not interfere with activity of the Apo-2. The tag polypeptide preferably also is fairly unique so that the antibody does not substantially cross-react with other epitopes. Suitable tag polypeptides generally have at least six amino acid residues and usually between about 8 to about 50 amino acid residues (preferably, between about 10 to about 20 residues).

"Isolated," when used to describe the various polypeptides disclosed herein, means polypeptide that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would typically interfere with



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diagnostic or therapeutic uses for the polypeptide, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In preferred embodiments, the polypeptide will be purified (1) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (2) to homogeneity by SDS-PAGE under non-reducing or reducing conditions using Coomassie blue or, preferably, silver stain. Isolated polypeptide includes polypeptide *in situ* within recombinant cells, since at least one component of the Apo-2 natural environment will not be present. Ordinarily, however, isolated polypeptide will be prepared by at least one purification step.

An "isolated" Apo-2 nucleic acid molecule is a nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the Apo-2 nucleic acid. An isolated Apo-2 nucleic acid molecule is other than in the form or setting in which it is found in nature. Isolated Apo-2 nucleic acid molecules therefore are distinguished from the Apo-2 nucleic acid molecule as it exists in natural cells. However, an isolated Apo-2 nucleic acid molecule includes Apo-2 nucleic acid molecules contained in cells that ordinarily express Apo-2 where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein

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that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

The term "antibody" is used in the broadest sense and specifically covers single anti-Apo-2 monoclonal antibodies (including agonist, antagonist, and neutralizing antibodies) and anti-Apo-2 antibody compositions with polypepitopic specificity.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally-occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen.

The monoclonal antibodies herein include hybrid and recombinant antibodies produced by splicing a variable (including hypervariable) domain of an anti-Apo-2 antibody with a constant domain (e.g. "humanized" antibodies), or a light chain with a heavy chain, or a chain from one species with a chain from another species, or fusions with heterologous proteins, regardless of species of origin or immunoglobulin class or subclass designation, as well as antibody fragments (e.g., Fab, F(ab')<sub>2</sub>, and Fv), so long as they exhibit the desired biological activity. See, e.g. U.S.

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Pat. No. 4,816,567 and Mage et al., in Monoclonal Antibody Production Techniques and Applications, pp.79-97 (Marcel Dekker, Inc.: New York, 1987).

Thus, the modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler and Milstein, Nature, 256:495 (1975), or may be made by recombinant DNA methods such as described in U.S. Pat. No. 4,816,567. The "monoclonal antibodies" may also be isolated from phage libraries generated using the techniques described in McCafferty et al., Nature, 348:552-554 (1990), for example.

"Humanized" forms of non-human (e.g. murine) antibodies are specific chimeric immunoglobulins, immunoglobulin chains, or fragments thereof (such as Fv, Fab, Fab', F(ab')<sub>2</sub> or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat, or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, the humanized antibody may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody

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optimally also will comprise at least a portion of an immunoglobulin constant region or domain (Fc), typically that of a human immunoglobulin.

"Biologically active" and "desired biological activity" for the purposes herein mean having the ability to modulate apoptosis (either in an agonistic or stimulating manner or in an antagonistic or blocking manner) in at least one type of mammalian cell *in vivo* or *ex vivo*.

The terms "apoptosis" and "apoptotic activity" are used in a broad sense and refer to the orderly or controlled form of cell death in mammals that is typically accompanied by one or more characteristic cell changes, including condensation of cytoplasm, loss of plasma membrane microvilli, segmentation of the nucleus, degradation of chromosomal DNA or loss of mitochondrial function. This activity can be determined and measured, for instance, by cell viability assays, FACS analysis or DNA electrophoresis, all of which are known in the art.

The terms "treating," "treatment," and "therapy" as used herein refer to curative therapy, prophylactic therapy, and preventative therapy.

The term "mammal" as used herein refers to any mammal classified as a mammal, including humans, cows, horses, dogs and cats. In a preferred embodiment of the invention, the mammal is a human.

## II. Compositions and Methods of the Invention

The present invention provides newly identified and isolated Apo-2 polypeptides. In particular, Applicants have identified and isolated various human Apo-2 polypeptides. The properties and characteristics of some of these Apo-2 polypeptides are described in further detail in the Examples below. Based upon the properties and characteristics of the Apo-2 polypeptides disclosed herein, it is Applicants' present belief that Apo-2 is a member of the TNFR family.

A description follows as to how Apo-2, as well as Apo-2

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chimeric molecules and anti-Apo-2 antibodies, may be prepared.

A. Preparation of Apo-2

The description below relates primarily to production of Apo-2 by culturing cells transformed or transfected with a vector containing Apo-2 nucleic acid. It is of course, contemplated that alternative methods, which are well known in the art, may be employed to prepare Apo-2.

1. Isolation of DNA Encoding Apo-2

The DNA encoding Apo-2 may be obtained from any cDNA library prepared from tissue believed to possess the Apo-2 mRNA and to express it at a detectable level. Accordingly, human Apo-2 DNA can be conveniently obtained from a cDNA library prepared from human tissues, such as the bacteriophage libraries of human pancreas and kidney cDNA described in Example 1. The Apo-2-encoding gene may also be obtained from a genomic library or by oligonucleotide synthesis.

Libraries can be screened with probes (such as antibodies to the Apo-2 or oligonucleotides of at least about 20-80 bases) designed to identify the gene of interest or the protein encoded by it. Screening the cDNA or genomic library with the selected probe may be conducted using standard procedures, such as described in Sambrook et al., Molecular Cloning: A Laboratory Manual (New York: Cold Spring Harbor Laboratory Press, 1989). An alternative means to isolate the gene encoding Apo-2 is to use PCR methodology [Sambrook et al., supra; Dieffenbach et al., PCR Primer: A Laboratory Manual (Cold Spring Harbor Laboratory Press, 1995)].

A preferred method of screening employs selected oligonucleotide sequences to screen cDNA libraries from various human tissues. Example 1 below describes techniques for screening a cDNA library. The oligonucleotide sequences selected as probes should be of sufficient length and sufficiently unambiguous that false positives are minimized. The oligonucleotide is preferably labeled such that it can be detected upon hybridization to DNA in the library being screened. Methods of labeling are well known in

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the art, and include the use of radiolabels like  $^{32}\text{P}$ -labeled ATP, biotinylation or enzyme labeling. Hybridization conditions, including moderate stringency and high stringency, are provided in Sambrook et al., supra.

5 Nucleic acid having all the protein coding sequence may be obtained by screening selected cDNA or genomic libraries using the deduced amino acid sequence disclosed herein for the first time, and, if necessary, using conventional primer extension procedures as described in Sambrook et al., supra, to detect  
10 precursors and processing intermediates of mRNA that may not have been reverse-transcribed into cDNA.

Apo-2 variants can be prepared by introducing appropriate nucleotide changes into the Apo-2 DNA, or by synthesis of the desired Apo-2 polypeptide. Those skilled in the art will  
15 appreciate that amino acid changes may alter post-translational processes of the Apo-2, such as changing the number or position of glycosylation sites or altering the membrane anchoring characteristics.

20 Variations in the native full-length sequence Apo-2 or in various domains of the Apo-2 described herein, can be made, for example, using any of the techniques and guidelines for conservative and non-conservative mutations set forth, for instance, in U.S. Pat. No. 5,364,934. Variations may be a substitution, deletion or insertion of one or more codons encoding  
25 the Apo-2 that results in a change in the amino acid sequence of the Apo-2 as compared with the native sequence Apo-2. Optionally the variation is by substitution of at least one amino acid with any other amino acid in one or more of the domains of the Apo-2 molecule. The variations can be made using methods known in the  
30 art such as oligonucleotide-mediated (site-directed) mutagenesis, alanine scanning, and PCR mutagenesis. Site-directed mutagenesis [Carter et al., Nucl. Acids Res., 13:4331 (1986); Zoller et al., Nucl. Acids Res., 10:6487 (1987)], cassette mutagenesis [Wells et al., Gene, 34:315 (1985)], restriction selection mutagenesis [Wells  
35 et al., Philos. Trans. R. Soc. London SerA, 317:415 (1986)] or

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other known techniques can be performed on the cloned DNA to produce the Apo-2 variant DNA.

5 Scanning amino acid analysis can also be employed to identify one or more amino acids along a contiguous sequence which are involved in the interaction with a particular ligand or receptor. Among the preferred scanning amino acids are relatively small, neutral amino acids. Such amino acids include alanine, glycine, serine, and cysteine. Alanine is the preferred scanning amino acid among this group because it eliminates the side-chain beyond the beta-carbon and is less likely to alter the main-chain conformation of the variant. Alanine is also preferred because it is the most common amino acid. Further, it is frequently found in both buried and exposed positions [Creighton, The Proteins, (W.H. Freeman & Co., N.Y.); Chothia, J. Mol. Biol., 150:1 (1976)]. If 10 alanine substitution does not yield adequate amounts of variant, an isoteric amino acid can be used.

Once selected Apo-2 variants are produced, they can be contacted with, for instance, Apo-2L, and the interaction, if any, can be determined. The interaction between the Apo-2 variant and Apo-2L can be measured by an *in vitro* assay, such as described in the Examples below. While any number of analytical measurements can be used to compare activities and properties between a native sequence Apo-2 and an Apo-2 variant, a convenient one for binding is the dissociation constant  $K_d$  of the complex formed between the Apo-2 variant and Apo-2L as compared to the  $K_d$  for the native sequence Apo-2. Generally, a \_\_\_\_\_-fold increase or decrease in  $K_d$  per substituted residue indicates that the substituted residue(s) is active in the interaction of the native sequence Apo-2 with the Apo-2L.

30 Avi, I'd like to particularly point out which domains in which mutagenesis would be preferable/not preferable. For instance, would it be better if the cysteine rich domains were conserved and not mutated? What about the ECD and death domain? If we consider the % homology of Apo-2 to other sequences, say DR4, would it be better to mutate residues which are not conserved? I know that some of 35

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these may not have clear cut answers but I'm trying to get some scope here on how to describe as many possible variants as we can.

Hence, representative sites in the Apo-2 sequence suitable for mutagenesis would include residues \_\_\_\_\_

## 2. Insertion of Nucleic Acid into A Replicable Vector

The nucleic acid (e.g., cDNA or genomic DNA) encoding Apo-2 may be inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. Various vectors are publicly available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence, each of which is described below.

### (i) Signal Sequence Component

The Apo-2 may be produced recombinantly not only directly, but also as a fusion polypeptide with a heterologous polypeptide, which may be a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. In general, the signal sequence may be a component of the vector, or it may be a part of the Apo-2 DNA that is inserted into the vector. The heterologous signal sequence selected preferably is one that is recognized and processed (i.e., cleaved by a signal peptidase) by the host cell. The signal sequence may be a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion the signal sequence may be, e.g., the yeast invertase leader, alpha factor leader (including *Saccharomyces* and *Kluyveromyces*  $\alpha$ -factor leaders, the latter described in U.S. Pat. No. 5,010,182), or acid phosphatase leader, the *C. albicans* glucoamylase leader (EP 362,179 published 4 April 1990), or the signal described in WO 90/13646 published 15 November 1990. In mammalian cell expression the native Apo-2 presequence that normally directs insertion of Apo-2

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in the cell membrane of human cells *in vivo* is satisfactory, although other mammalian signal sequences may be used to direct secretion of the protein, such as signal sequences from secreted polypeptides of the same or related species, as well as viral secretory leaders, for example, the herpes simplex glycoprotein D signal.

The DNA for such precursor region is preferably ligated in reading frame to DNA encoding Apo-2.

(ii) Origin of Replication Component

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2 $\mu$  plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used because it contains the early promoter).

Most expression vectors are "shuttle" vectors, i.e., they are capable of replication in at least one class of organisms but can be transfected into another organism for expression. For example, a vector is cloned in *E. coli* and then the same vector is transfected into yeast or mammalian cells for expression even though it is not capable of replicating independently of the host cell chromosome.

DNA may also be amplified by insertion into the host genome. This is readily accomplished using *Bacillus* species as hosts, for example, by including in the vector a DNA sequence that is complementary to a sequence found in *Bacillus* genomic DNA. Transfection of *Bacillus* with this vector results in homologous

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recombination with the genome and insertion of Apo-2 DNA. However, the recovery of genomic DNA encoding Apo-2 is more complex than that of an exogenously replicated vector because restriction enzyme digestion is required to excise the Apo-2 DNA.

(iii) Selection Gene Component

Expression and cloning vectors typically contain a selection gene, also termed a selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g., the gene encoding D-alanine racemase for *Bacilli*.

One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene produce a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin [Southern et al., J. Molec. Appl. Genet., 1:327 (1982)], mycophenolic acid (Mulligan et al., Science, 209:1422 (1980)) or hygromycin [Sugden et al., Mol. Cell. Biol., 5:410-413 (1985)]. The three examples given above employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug G418 or neomycin (geneticin), xgpt (mycophenolic acid), or hygromycin, respectively.

Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the Apo-2 nucleic acid, such as DHFR or thymidine kinase. The mammalian cell transformants are placed under selection pressure that only the transformants are uniquely adapted to survive by virtue of having taken up the marker. Selection pressure is imposed by culturing the transformants under conditions in which the concentration of selection agent in the

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medium is successively changed, thereby leading to amplification of both the selection gene and the DNA that encodes Apo-2. Amplification is the process by which genes in greater demand for the production of a protein critical for growth are reiterated in tandem within the chromosomes of successive generations of recombinant cells. Increased quantities of Apo-2 are synthesized from the amplified DNA. Other examples of amplifiable genes include metallothionein-I and -II, adenosine deaminase, and ornithine decarboxylase.

Cells transformed with the DHFR selection gene may first be identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub et al., Proc. Natl. Acad. Sci. USA, 77:4216 (1980). The transformed cells are then exposed to increased levels of methotrexate. This leads to the synthesis of multiple copies of the DHFR gene, and, concomitantly, multiple copies of other DNA comprising the expression vectors, such as the DNA encoding Apo-2. This amplification technique can be used with any otherwise suitable host, e.g., ATCC No. CCL61 CHO-K1, notwithstanding the presence of endogenous DHFR if, for example, a mutant DHFR gene that is highly resistant to Mtx is employed (EP 117,060).

Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding Apo-2, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3'-phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, e.g., kanamycin, neomycin, or G418. See U.S. Patent No. 4,965,199.

A suitable selection gene for use in yeast is the *trp1* gene present in the yeast plasmid YRp7 [Stinchcomb et al., Nature, 282:39 (1979); Kingsman et al., Gene, 7:141 (1979); Tschemper et

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al., Gene, 10:157 (1980)]. The *trp1* gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1 [Jones, Genetics, 85:12 (1977)]. The presence of the *trp1* lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, *Leu2*-deficient yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the *Leu2* gene.

In addition, vectors derived from the 1.6  $\mu$ m circular plasmid pKD1 can be used for transformation of *Kluyveromyces* yeasts [Bianchi et al., Curr. Genet., 12:185 (1987)]. More recently, an expression system for large-scale production of recombinant calf chymosin was reported for *K. lactis* [Van den Berg, Bio/Technology, 8:135 (1990)]. Stable multi-copy expression vectors for secretion of mature recombinant human serum albumin by industrial strains of *Kluyveromyces* have also been disclosed [Fleer et al., Bio/Technology, 9:968-975 (1991)].

#### (iv) Promoter Component

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the Apo-2 nucleic acid sequence. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of particular nucleic acid sequence, such as the Apo-2 nucleic acid sequence, to which they are operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, e.g., the presence or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These promoters are operably linked to Apo-2 encoding DNA by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector. Both the native Apo-2 promoter sequence

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and many heterologous promoters may be used to direct amplification and/or expression of the Apo-2 DNA.

Promoters suitable for use with prokaryotic hosts include the  $\beta$ -lactamase and lactose promoter systems [Chang et al., Nature, 275:615 (1978); Goeddel et al., Nature, 281:544 (1979)], alkaline phosphatase, a tryptophan (trp) promoter system [Goeddel, Nucleic Acids Res., 8:4057 (1980); EP 36,776], and hybrid promoters such as the tac promoter [deBoer et al., Proc. Natl. Acad. Sci. USA, 80:21-25 (1983)]. However, other known bacterial promoters are suitable. Their nucleotide sequences have been published, thereby enabling a skilled worker operably to ligate them to DNA encoding Apo-2 [Siebenlist et al., Cell, 20:269 (1980)] using linkers or adaptors to supply any required restriction sites. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding Apo-2.

Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CXCAAT region where X may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into eukaryotic expression vectors.

Examples of suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase [Hitzeman et al., J. Biol. Chem., 255:2073 (1980)] or other glycolytic enzymes [Hess et al., J. Adv. Enzyme Reg., 7:149 (1968); Holland, Biochemistry, 17:4900 (1978)], such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by

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growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in EP 73,657. Yeast enhancers also are advantageously used with yeast promoters.

Apo-2 transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published 5 July 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40), from heterologous mammalian promoters, e.g., the actin promoter or an immunoglobulin promoter, from heat-shock promoters, and from the promoter normally associated with the Apo-2 sequence, provided such promoters are compatible with the host cell systems.

The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication [Fiers et al., Nature, 273:113 (1978); Mulligan and Berg, Science, 209:1422-1427 (1980); Pavlakis et al., Proc. Natl. Acad. Sci. USA, 78:7398-7402 (1981)]. The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment [Greenaway et al., Gene, 18:355-360 (1982)]. A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. Patent No. 4,419,446. A modification of this system is described in U.S. Patent No. 4,601,978 [See also Gray et al., Nature, 295:503-508 (1982) on expressing cDNA encoding immune interferon in monkey cells; Reyes et al., Nature, 297:598-601 (1982) on expression of human  $\beta$ -interferon cDNA in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus; Canaani and Berg, Proc. Natl. Acad. Sci. USA 79:5166-5170 (1982) on expression of the human

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interferon  $\beta$ 1 gene in cultured mouse and rabbit cells; and Gorman et al., Proc. Natl. Acad. Sci. USA, 79:6777-6781 (1982) on expression of bacterial CAT sequences in CV-1 monkey kidney cells, chicken embryo fibroblasts, Chinese hamster ovary cells, HeLa cells, and mouse NIH-3T3 cells using the Rous sarcoma virus long terminal repeat as a promoter].

(v) Enhancer Element Component

Transcription of a DNA encoding the Apo-2 of this invention by higher eukaryotes may be increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp, that act on a promoter to increase its transcription. Enhancers are relatively orientation and position independent, having been found 5' [Laimins et al., Proc. Natl. Acad. Sci. USA, 78:993 (1981)] and 3' [Lusky et al., Mol. Cell Bio., 3:1108 (1983)] to the transcription unit, within an intron [Banerji et al., Cell, 33:729 (1983)], as well as within the coding sequence itself [Osborne et al., Mol. Cell Bio., 4:1293 (1984)]. Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin,  $\alpha$ -fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270); the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, Nature, 297:17-18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to the Apo-2 coding sequence, but is preferably located at a site 5' from the promoter.

(vi) Transcription Termination Component

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs.

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These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding Apo-2.

(vii) Construction and Analysis of Vectors

Construction of suitable vectors containing one or more of the above-listed components employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and re-ligated in the form desired to generate the plasmids required.

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures can be used to transform *E. coli* K12 strain 294 (ATCC 31,446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced by the method of Messing et al., Nucleic Acids Res., 9:309 (1981) or by the method of Maxam et al., Methods in Enzymology, 65:499 (1980).

(viii) Transient Expression Vectors

Expression vectors that provide for the transient expression in mammalian cells of DNA encoding Apo-2 may be employed. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector [Sambrook et al., *supra*]. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of such polypeptides for desired biological or physiological properties. Thus, transient expression systems are particularly useful in the invention for purposes of identifying Apo-2 variants.

(ix) Suitable Exemplary Vertebrate Cell Vectors

Other methods, vectors, and host cells suitable for adaptation to the synthesis of Apo-2 in recombinant vertebrate cell

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culture are described in Gething et al., Nature, 293:620-625 (1981); Mantei et al., Nature, 281:40-46 (1979); EP 117,060; and EP 117,058.

### 3. Selection and Transformation of Host Cells

Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include but are not limited to eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as *Escherichia*, e.g., *E. coli*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Proteus*, *Salmonella*, e.g., *Salmonella typhimurium*, *Serratia*, e.g., *Serratia marcescans*, and *Shigella*, as well as *Bacilli* such as *B. subtilis* and *B. licheniformis* (e.g., *B. licheniformis* 41P disclosed in DD 266,710 published 12 April 1989), *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*. Preferably, the host cell should secrete minimal amounts of proteolytic enzymes.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for Apo-2-encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein.

Suitable host cells for the expression of glycosylated Apo-2 are derived from multicellular organisms. Such host cells are capable of complex processing and glycosylation activities. In principle, any higher eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified [See, e.g., Luckow et al., Bio/Technology, 6:47-55 (1988); Miller et al., in Genetic Engineering, Setlow et al., eds., Vol. 8 (Plenum Publishing, 1986), pp. 277-279; and Maeda et al., Nature, 315:592-594 (1985)]. A

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variety of viral strains for transfection are publicly available, e.g., the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV.

Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can be utilized as hosts. Typically, plant cells are transfected by incubation with certain strains of the bacterium *Agrobacterium tumefaciens*. During incubation of the plant cell culture with *A. tumefaciens*, the DNA encoding the Apo-2 can be transferred to the plant cell host such that it is transfected, and will, under appropriate conditions, express the Apo-2-encoding DNA. In addition, regulatory and signal sequences compatible with plant cells are available, such as the nopaline synthase promoter and polyadenylation signal sequences [Depicker et al., J. Mol. Appl. Gen., 1:561 (1982)]. In addition, DNA segments isolated from the upstream region of the T-DNA 780 gene are capable of activating or increasing transcription levels of plant-expressible genes in recombinant DNA-containing plant tissue [EP 321,196 published 21 June 1989].

Propagation of vertebrate cells in culture (tissue culture) is also well known in the art [See, e.g., Tissue Culture, Academic Press, Kruse and Patterson, editors (1973)]. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., J. Gen Virol., 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, Proc. Natl. Acad. Sci. USA, 77:4216 (1980)); mouse sertoli cells (TM4, Mather, Biol. Reprod., 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., Annals N.Y. Acad. Sci., 383:44-68 (1982)); MRC 5

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cells; and FS4 cells.

Host cells are transfected and preferably transformed with the above-described expression or cloning vectors for Apo-2 production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example,  $\text{CaPO}_4$  and electroporation. Successful transfection is generally recognized when any indication of the operation of this vector occurs within the host cell.

Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in Sambrook et al., supra, or electroporation is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. Infection with *Agrobacterium tumefaciens* is used for transformation of certain plant cells, as described by Shaw et al., Gene, 23:315 (1983) and WO 89/05859 published 29 June 1989. In addition, plants may be transfected using ultrasound treatment as described in WO 91/00358 published 10 January 1991.

For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb, Virology, 52:456-457 (1978) is preferred. General aspects of mammalian cell host system transformations have been described in U.S. Pat. No. 4,399,216. Transformations into yeast are typically carried out according to the method of Van Solingen et al., J. Bact., 130:946 (1977) and Hsiao et al., Proc. Natl. Acad. Sci. (USA), 76:3829 (1979). However, other methods for introducing DNA into cells, such as by nuclear microinjection, electroporation, bacterial protoplast fusion with intact cells, or polycations, e.g.,

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polybrene, polyornithine, may also be used. For various techniques for transforming mammalian cells, see Keown et al., Methods in Enzymology, 185:527-537 (1990) and Mansour et al., Nature, 336:348-352 (1988).

5                   4.    Culturing the Host Cells

Prokaryotic cells used to produce Apo-2 may be cultured in suitable media as described generally in Sambrook et al., supra.

10                   The mammalian host cells used to produce Apo-2 may be cultured in a variety of media. Examples of commercially available media include Ham's F10 (Sigma), Minimal Essential Medium ("MEM", Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ("DMEM", Sigma). Any such media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

25                   In general, principles, protocols, and practical techniques for maximizing the productivity of mammalian cell cultures can be found in Mammalian Cell Biotechnology: a Practical Approach, M. Butler, ed. (IRL Press, 1991).

30                   The host cells referred to in this disclosure encompass cells in culture as well as cells that are within a host animal.

5.    Detecting Gene Amplification/Expression

35                   Gene amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA [Thomas, Proc. Natl. Acad. Sci. USA, 77:5201-5205 (1980)], dot blotting (DNA

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analysis), or *in situ* hybridization, using an appropriately labeled probe, based on the sequences provided herein. Various labels may be employed, most commonly radioisotopes, and particularly  $^{32}\text{P}$ . However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionucleotides, fluorescers or enzymes. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of cells or tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. With immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and fixation, followed by reaction with labeled antibodies specific for the gene product coupled, where the labels are usually visually detectable, such as enzymatic labels, fluorescent labels, or luminescent labels.

Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared in any mammal. Conveniently, the antibodies may be prepared against a native sequence Apo-2 polypeptide or against a synthetic peptide based on the DNA sequences provided herein or against exogenous sequence fused to Apo-2 DNA and encoding a specific antibody epitope.

#### 6. Purification of Apo-2 Polypeptide

Forms of Apo-2 may be recovered from culture medium or from host cell lysates. If the Apo-2 is membrane-bound, it can be released from the membrane using a suitable detergent solution (e.g. Triton-X 100) or its extracellular domain may be released by

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enzymatic cleavage.

When Apo-2 is produced in a recombinant cell other than one of human origin, the Apo-2 is free of proteins or polypeptides of human origin. However, it may be desired to purify Apo-2 from recombinant cell proteins or polypeptides to obtain preparations that are substantially homogeneous as to Apo-2. As a first step, the culture medium or lysate may be centrifuged to remove particulate cell debris. Apo-2 thereafter is purified from contaminant soluble proteins and polypeptides, with the following procedures being exemplary of suitable purification procedures: by fractionation on an ion-exchange column; ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; and protein A Sepharose columns to remove contaminants such as IgG.

Apo-2 variants in which residues have been deleted, inserted, or substituted can be recovered in the same fashion as native sequence Apo-2, taking account of changes in properties occasioned by the variation. For example, preparation of an Apo-2 fusion with another protein or polypeptide, e.g., a bacterial or viral antigen, immunoglobulin sequence, or receptor sequence, may facilitate purification; an immunoaffinity column containing antibody to the sequence can be used to adsorb the fusion polypeptide. Other types of affinity matrices also can be used.

A protease inhibitor such as phenyl methyl sulfonyl fluoride (PMSF) also may be useful to inhibit proteolytic degradation during purification, and antibiotics may be included to prevent the growth of adventitious contaminants. One skilled in the art will appreciate that purification methods suitable for native sequence Apo-2 may require modification to account for changes in the character of Apo-2 or its variants upon expression in recombinant cell culture.

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7. Covalent Modifications of Apo-2 Polypeptides

Covalent modifications of Apo-2 are included within the scope of this invention. One type of covalent modification of the Apo-2 is introduced into the molecule by reacting targeted amino acid residues of the Apo-2 with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues of the Apo-2.

Derivatization with bifunctional agents is useful for crosslinking Apo-2 to a water-insoluble support matrix or surface for use in the method for purifying anti-Apo-2 antibodies, and vice-versa. Derivatization with one or more bifunctional agents will also be useful for crosslinking Apo-2 molecules to generate Apo-2 dimers. Such dimers may increase binding avidity and extend half-life of the molecule *in vivo*. Commonly used crosslinking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidyl-propionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)-dithio]propioimide yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Patent Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

Other modifications include deamidation of glutaminyl and asparaginyl residues to the corresponding glutamyl and aspartyl residues, respectively, hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the  $\alpha$ -amino groups of lysine, arginine, and histidine side chains [T.E. Creighton, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)], acetylation of the N-terminal amine, and amidation of any

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C-terminal carboxyl group. The modified forms of the residues fall within the scope of the present invention.

Another type of covalent modification of the Apo-2 polypeptide included within the scope of this invention comprises altering the native glycosylation pattern of the polypeptide. "Altering the native glycosylation pattern" is intended for purposes herein to mean deleting one or more carbohydrate moieties found in native sequence Apo-2, and/or adding one or more glycosylation sites that are not present in the native sequence Apo-2.

Glycosylation of polypeptides is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-aceylgalactosamine, galactose, or xylose to a hydroxylamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Addition of glycosylation sites to the Apo-2 polypeptide may be accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the native sequence Apo-2 (for O-linked glycosylation sites). The Apo-2 amino acid sequence may optionally be altered through changes at the DNA level, particularly by mutating the DNA encoding the Apo-2 polypeptide at preselected bases such that codons are generated that will translate into the desired amino acids. The DNA mutation(s) may be made using methods described above and in U.S. Pat. No. 5,364,934, supra.

Another means of increasing the number of carbohydrate

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moieties on the Apo-2 polypeptide is by chemical or enzymatic coupling of glycosides to the polypeptide. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. These methods are described in WO 87/05330 published 11 September 1987, and in Aplin and Wriston, CRC Crit. Rev. Biochem., pp. 259-306 (1981).

Removal of carbohydrate moieties present on the Apo-2 polypeptide may be accomplished chemically or enzymatically or by mutational substitution of codons encoding for amino acid residues that serve as targets for glycosylation. For instance, chemical deglycosylation by exposing the polypeptide to the compound trifluoromethanesulfonic acid, or an equivalent compound can result in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the polypeptide intact. Chemical deglycosylation is described by Hakimuddin, et al., Arch. Biochem. Biophys., 259:52 (1987) and by Edge et al., Anal. Biochem., 118:131 (1981). Enzymatic cleavage of carbohydrate moieties on polypeptides can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura et al., Meth. Enzymol., 138:350 (1987).

Glycosylation at potential glycosylation sites may be prevented by the use of the compound tunicamycin as described by Duskin et al., J. Biol. Chem., 257:3105 (1982). Tunicamycin blocks the formation of protein-N-glycoside linkages.

Another type of covalent modification of Apo-2 comprises linking the Apo-2 polypeptide to one of a variety of nonproteinaceous polymers, e.g., polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

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8. Apo-2 Chimeras

The present invention also provides chimeric molecules comprising Apo-2 fused to another, heterologous polypeptide or amino acid sequence.

5 In one embodiment, the chimeric molecule comprises a fusion of the Apo-2 with a tag polypeptide which provides an epitope to which an anti-tag antibody can selectively bind. The epitope tag is generally placed at the amino- or carboxyl- terminus of the Apo-2. The presence of such epitope-tagged forms of the  
10 Apo-2 can be detected using an antibody against the tag polypeptide. Also, provision of the epitope tag enables the Apo-2 to be readily purified by affinity purification using an anti-tag antibody or another type of affinity matrix that binds to the epitope tag.

15 Various tag polypeptides and their respective antibodies are well known in the art. Examples include the flu HA tag polypeptide and its antibody 12CA5 [Field et al., Mol. Cell. Biol., 8:2159-2165 (1988)]; the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto [Evan et al., Molecular and Cellular Biology, 5:3610-3616 (1985)]; and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody [Paborsky et al., Protein Engineering, 3(6):547-553 (1990)]. Other tag polypeptides include the Flag-peptide [Hopp et al., BioTechnology, 6:1204-1210 (1988)]; the KT3 epitope peptide [Martin et al., Science, 255:192-194  
20 (1992)]; an  $\alpha$ -tubulin epitope peptide [Skinner et al., J. Biol. Chem., 266:15163-15166 (1991)]; and the T7 gene 10 protein peptide tag [Lutz-Freyermuth et al., Proc. Natl. Acad. Sci. USA, 87:6393-6397 (1990)]. Once the tag polypeptide has been selected, an  
25 antibody thereto can be generated using the techniques disclosed herein.

30 Generally, epitope-tagged Apo-2 may be constructed and produced according to the methods described above. Epitope-tagged Apo-2 is also described in the Examples below. Apo-2-tag polypeptide fusions are preferably constructed by fusing the cDNA  
35 sequence encoding the Apo-2 portion in-frame to the tag polypeptide

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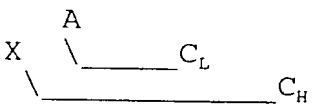
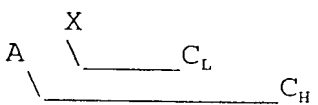
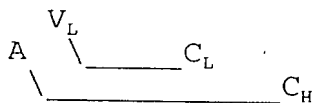
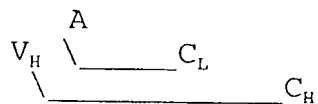
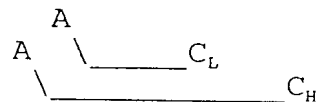
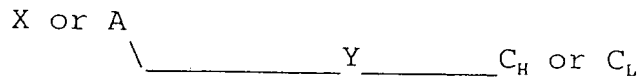
DNA sequence and expressing the resultant DNA fusion construct in appropriate host cells. Ordinarily, when preparing the Apo-2-tag polypeptide chimeras of the present invention, nucleic acid encoding the Apo-2 will be fused at its 3' end to nucleic acid encoding the N-terminus of the tag polypeptide, however 5' fusions are also possible. For example, a polyhistidine sequence of about 5 to about 10 histidine residues may be fused at the N-terminus or the C-terminus and used as a purification handle in affinity chromatography.

Epitope-tagged Apo-2 can be purified by affinity chromatography using the anti-tag antibody. The matrix to which the affinity antibody is attached may include, for instance, agarose, controlled pore glass or poly(styrenedivinyl)benzene. The epitope-tagged Apo-2 can then be eluted from the affinity column using techniques known in the art.

In another embodiment, the chimeric molecule comprises an Apo-2 polypeptide fused to an immunoglobulin sequence. The chimeric molecule may also comprise a particular domain sequence of Apo-2, such as the extracellular domain sequence of native Apo-2 fused to an immunoglobulin sequence. This includes chimeras in monomeric, homo- or heteromultimeric, and particularly homo- or heterodimeric, or -tetrameric forms; optionally, the chimeras may be in dimeric forms or homodimeric heavy chain forms. Generally, these assembled immunoglobulins will have known unit structures as represented by the following diagrams.

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A basic four chain structural unit is the form in which IgG, IgD, and IgE exist. A four chain unit is repeated in the higher molecular weight immunoglobulins; IgM generally exists as a pentamer of basic four-chain units held together by disulfide bonds. IgA globulin, and occasionally IgG globulin, may also exist in a multimeric form in serum. In the case of multimers, each four chain unit may be the same or different.

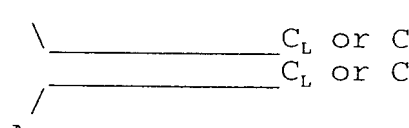
The following diagrams depict some exemplary monomer, homo- and heterodimer and homo- and heteromultimer structures. These diagrams are merely illustrative, and the chains of the

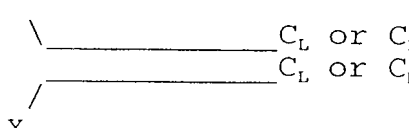
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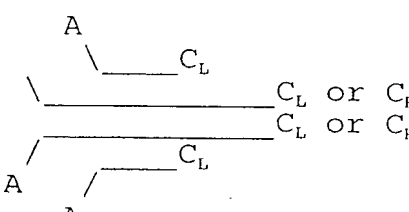
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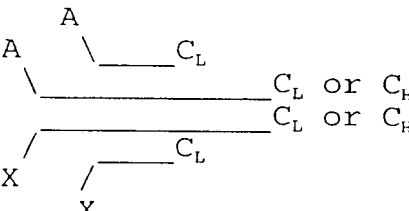
multimers are believed to be disulfide bonded in the same fashion as native immunoglobulins.

monomer: A \_\_\_\_\_ C<sub>L</sub> or C<sub>H</sub>

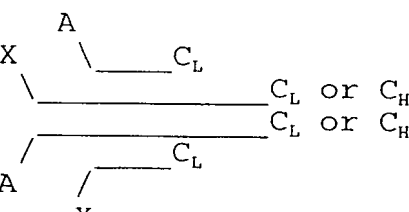
homodimer: A 

heterodimer: A 

homotetramer: A 

heterotetramer: A 

and



In the foregoing diagrams, "A" means an Apo-2 sequence or an Apo-2 sequence fused to a heterologous sequence; X is an additional agent, which may be the same as A or different, a

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portion of an immunoglobulin superfamily member such as a variable region or a variable region-like domain, including a native or chimeric immunoglobulin variable region, a toxin such as a pseudomonas exotoxin or ricin, or a sequence functionally binding to another protein, such as other cytokines (i.e., IL-1, interferon- $\gamma$ ) or cell surface molecules (i.e., NGFR, CD40, OX40, Fas antigen, T2 proteins of Shope and myxoma poxviruses), or a polypeptide therapeutic agent not otherwise normally associated with a constant domain; Y is a linker or another receptor sequence; and  $V_L$ ,  $V_H$ ,  $C_L$  and  $C_H$  represent light or heavy chain variable or constant domains of an immunoglobulin. Structures comprising at least one CRD of an Apo-2 sequence as "A" and another cell-surface protein having a repetitive pattern of CRDs (such as TNFR) as "X" are specifically included.

It will be understood that the above diagrams are merely exemplary of the possible structures of the chimeras of the present invention, and do not encompass all possibilities. For example, there might desirably be several different "A"s, "X"s, or "Y"s in any of these constructs. Also, the heavy or light chain constant domains may be originated from the same or different immunoglobulins. All possible permutations of the illustrated and similar structures are all within the scope of the invention herein.

In general, the chimeric molecules can be constructed in a fashion similar to chimeric antibodies in which a variable domain from an antibody of one species is substituted for the variable domain of another species. See, for example, EP 0 125 023; EP 173,494; Munro, Nature, 312:597 (13 December 1984); Neuberger et al., Nature, 312:604-608 (13 December 1984); Sharon et al., Nature, 309:364-367 (24 May 1984); Morrison et al., Proc. Nat'l. Acad. Sci. USA, 81:6851-6855 (1984); Morrison et al., Science, 229:1202-1207 (1985); Boulianne et al., Nature, 312:643-646 (13 December 1984); Capon et al., Nature, 337:525-531 (1989); Traunecker et al., Nature, 339:68-70 (1989).

Alternatively, the chimeric molecules may be constructed

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as follows. The DNA including a region encoding the desired sequence, such as an Apo-2 and/or TNFR sequence, is cleaved by a restriction enzyme at or proximal to the 3' end of the DNA encoding the immunoglobulin-like domain(s) and at a point at or near the DNA encoding the N-terminal end of the Apo-2 or TNFR polypeptide (where use of a different leader is contemplated) or at or proximal to the N-terminal coding region for TNFR (where the native signal is employed). This DNA fragment then is readily inserted proximal to DNA encoding an immunoglobulin light or heavy chain constant region and, if necessary, the resulting construct tailored by deletional mutagenesis. Preferably, the Ig is a human immunoglobulin when the chimeric molecule is intended for *in vivo* therapy for humans. DNA encoding immunoglobulin light or heavy chain constant regions is known or readily available from cDNA libraries or is synthesized. See for example, Adams et al., Biochemistry, 19:2711-2719 (1980); Gough et al., Biochemistry, 19:2702-2710 (1980); Dolby et al., Proc. Natl. Acad. Sci. USA, 77:6027-6031 (1980); Rice et al., Proc. Natl. Acad. Sci., 79:7862-7865 (1982); Falkner et al., Nature, 298:286-288 (1982); and Morrison et al., Ann. Rev. Immunol., 2:239-256 (1984).

Further details of how to prepare such fusions are found in publications concerning the preparation of immunoadhesins. Immunoadhesins in general, and CD4-Ig fusion molecules specifically are disclosed in WO 89/02922, published 6 April 1989). Molecules comprising the extracellular portion of CD4, the receptor for human immunodeficiency virus (HIV), linked to IgG heavy chain constant region are known in the art and have been found to have a markedly longer half-life and lower clearance than the soluble extracellular portion of CD4 [Capon et al., supra; Byrn et al., Nature, 344:667 (1990)]. The construction of specific chimeric TNFR-IgG molecules is also described in Ashkenazi et al. Proc. Natl. Acad. Sci., 88:10535-10539 (1991); Lesslauer et al. [J. Cell. Biochem. Supplement 15F, 1991, p. 115 (P 432)]; and Peppel and Beutler, J. Cell. Biochem. Supplement 15F, 1991, p. 118 (P 439)].

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B. Therapeutic and Non-therapeutic Uses for Apo-2

Apo-2, as disclosed in the present specification, can be employed therapeutically to induce apoptosis in mammalian cells. This therapy can be accomplished for instance, using *in vivo* or *ex vivo* gene therapy techniques and includes the use of the death domain sequences disclosed herein. The Apo-2 chimeric molecules (including the chimeric molecules containing the extracellular domain sequence of Apo-2) comprising immunoglobulin sequences can also be employed therapeutically to inhibit apoptosis or NF- $\kappa$ B induction.

The Apo-2 of the invention also has utility in non-therapeutic applications. Nucleic acid sequences encoding the Apo-2 may be used as a diagnostic for tissue-specific typing. For example, procedures like *in situ* hybridization, Northern and Southern blotting, and PCR analysis may be used to determine whether DNA and/or RNA encoding Apo-2 is present in the cell type(s) being evaluated. Apo-2 nucleic acid will also be useful for the preparation of Apo-2 by the recombinant techniques described herein.

The isolated Apo-2 may be used in quantitative diagnostic assays as a control against which samples containing unknown quantities of Apo-2 may be prepared. Apo-2 preparations are also useful in generating antibodies, as standards in assays for Apo-2 (e.g., by labeling Apo-2 for use as a standard in a radioimmunoassay, radioreceptor assay, or enzyme-linked immunoassay), in affinity purification techniques, and in competitive-type receptor binding assays when labeled with, for instance, radioiodine, enzymes, or fluorophores.

Modified forms of the Apo-2, such as the Apo-2-IgG chimeric molecules (immunoadhesins) described above, can be used as immunogens in producing anti-Apo-2 antibodies.

Nucleic acids which encode Apo-2 or its modified forms can also be used to generate either transgenic animals or "knock out" animals which, in turn, are useful in the development and screening of therapeutically useful reagents. A transgenic animal

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(e.g., a mouse or rat) is an animal having cells that contain a transgene, which transgene was introduced into the animal or an ancestor of the animal at a prenatal, e.g., an embryonic stage. A transgene is a DNA which is integrated into the genome of a cell from which a transgenic animal develops. In one embodiment, cDNA encoding Apo-2 or an appropriate sequence thereof can be used to clone genomic DNA encoding Apo-2 in accordance with established techniques and the genomic sequences used to generate transgenic animals that contain cells which express DNA encoding Apo-2. Methods for generating transgenic animals, particularly animals such as mice or rats, have become conventional in the art and are described, for example, in U.S. Patent Nos. 4,736,866 and 4,870,009. Typically, particular cells would be targeted for Apo-2 transgene incorporation with tissue-specific enhancers. Transgenic animals that include a copy of a transgene encoding Apo-2 introduced into the germ line of the animal at an embryonic stage can be used to examine the effect of increased expression of DNA encoding Apo-2. Such animals can be used as tester animals for reagents thought to confer protection from, for example, pathological conditions associated with excessive apoptosis. In accordance with this facet of the invention, an animal is treated with the reagent and a reduced incidence of the pathological condition, compared to untreated animals bearing the transgene, would indicate a potential therapeutic intervention for the pathological condition. In another embodiment, transgenic animals that carry a soluble form of Apo-2 such as the Apo-2 ECD or an immunoglobulin chimera of such form could be constructed to test the effect of chronic neutralization of Apo-2L, a ligand of Apo-2.

Alternatively, non-human homologues of Apo-2 can be used to construct an Apo-2 "knock out" animal which has a defective or altered gene encoding Apo-2 as a result of homologous recombination between the endogenous gene encoding Apo-2 and altered genomic DNA encoding Apo-2 introduced into an embryonic cell of the animal. For example, cDNA encoding Apo-2 can be used to clone genomic DNA encoding Apo-2 in accordance with established techniques. A

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portion of the genomic DNA encoding Apo-2 can be deleted or replaced with another gene, such as a gene encoding a selectable marker which can be used to monitor integration. Typically, several kilobases of unaltered flanking DNA (both at the 5' and 3' ends) are included in the vector [see e.g., Thomas and Capecchi, Cell, 51:503 (1987) for a description of homologous recombination vectors]. The vector is introduced into an embryonic stem cell line (e.g., by electroporation) and cells in which the introduced DNA has homologously recombined with the endogenous DNA are selected [see e.g., Li et al., Cell, 69:915 (1992)]. The selected cells are then injected into a blastocyst of an animal (e.g., a mouse or rat) to form aggregation chimeras [see e.g., Bradley, in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E. J. Robertson, ed. (IRL, Oxford, 1987), pp. 113-152]. A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term to create a "knock out" animal. Progeny harboring the homologously recombined DNA in their germ cells can be identified by standard techniques and used to breed animals in which all cells of the animal contain the homologously recombined DNA. Knockout animals can be characterized for instance, for their ability to defend against certain pathological conditions and for their development of pathological conditions due to absence of the Apo-2 polypeptide, including for example, development of tumors.

### C. Anti-Apo-2 Antibody Preparation

The present invention further provides anti-Apo-2 antibodies. Antibodies against Apo-2 may be prepared as follows. Exemplary antibodies include polyclonal, monoclonal, humanized, bispecific, and heteroconjugate antibodies.

#### 1. Polyclonal Antibodies

The Apo-2 antibodies may comprise polyclonal antibodies. Methods of preparing polyclonal antibodies are known to the skilled artisan. Polyclonal antibodies can be raised in a mammal, for example, by one or more injections of an immunizing agent and, if

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desired, an adjuvant. Typically, the immunizing agent and/or adjuvant will be injected in the mammal by multiple subcutaneous or intraperitoneal injections. The immunizing agent may include the Apo-2 polypeptide or a fusion protein thereof. An example of a suitable immunizing agent is a Apo-2-IgG fusion protein or chimeric molecule (including an Apo-2 ECD-IgG fusion protein). Cells expressing Apo-2 at their surface may also be employed. It may be useful to conjugate the immunizing agent to a protein known to be immunogenic in the mammal being immunized. Examples of such immunogenic proteins which may be employed include but are not limited to keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, and soybean trypsin inhibitor. An aggregating agent such as alum may also be employed to enhance the mammal's immune response. Examples of adjuvants which may be employed include Freund's complete adjuvant and MPL-TDM adjuvant (monophosphoryl Lipid A, synthetic trehalose dicorynomycolate). The immunization protocol may be selected by one skilled in the art without undue experimentation. The mammal can then be bled, and the serum assayed for antibody titer. If desired, the mammal can be boosted until the antibody titer increases or plateaus.

2. Monoclonal Antibodies

The Apo-2 antibodies may, alternatively, be monoclonal antibodies. Monoclonal antibodies may be prepared using hybridoma methods, such as those described by Kohler and Milstein, supra. In a hybridoma method, a mouse, hamster, or other appropriate host animal, is typically immunized (such as described above) with an immunizing agent to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the immunizing agent. Alternatively, the lymphocytes may be immunized *in vitro*.

The immunizing agent will typically include the Apo-2 polypeptide or a fusion protein thereof. An example of a suitable immunizing agent is a Apo-2-IgG fusion protein or chimeric molecule. Cells expressing Apo-2 at their surface may also be employed. Generally, either peripheral blood lymphocytes ("PBLs")

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are used if cells of human origin are desired, or spleen cells or lymph node cells are used if non-human mammalian sources are desired. The lymphocytes are then fused with an immortalized cell line using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell [Goding, Monoclonal Antibodies: Principles and Practice, Academic Press, (1986) pp. 59-103]. Immortalized cell lines are usually transformed mammalian cells, particularly myeloma cells of rodent, bovine and human origin. Usually, rat or mouse myeloma cell lines are employed. The hybridoma cells may be cultured in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, immortalized cells. For example, if the parental cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine ("HAT medium"), which substances prevent the growth of HGPRT-deficient cells.

Preferred immortalized cell lines are those that fuse efficiently, support stable high level expression of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. More preferred immortalized cell lines are murine myeloma lines, which can be obtained, for instance, from the Salk Institute Cell Distribution Center, San Diego, California and the American Type Culture Collection, Rockville, Maryland. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies [Kozbor, J. Immunol., 133:3001 (1984); Brodeur et al., Monoclonal Antibody Production Techniques and Applications, Marcel Dekker, Inc., New York, (1987) pp. 51-63].

The culture medium in which the hybridoma cells are cultured can then be assayed for the presence of monoclonal antibodies directed against Apo-2. Preferably, the binding specificity of monoclonal antibodies produced by the hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked

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immunoabsorbent assay (ELISA). Such techniques and assays are known in the art. The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson and Pollard, Anal. Biochem., 107:220 (1980).

5 After the desired hybridoma cells are identified, the clones may be subcloned by limiting dilution procedures and grown by standard methods [Goding, supra]. Suitable culture media for this purpose include, for example, Dulbecco's Modified Eagle's Medium and RPMI-1640 medium. Alternatively, the hybridoma cells  
10 may be grown *in vivo* as ascites in a mammal.

The monoclonal antibodies secreted by the subclones may be isolated or purified from the culture medium or ascites fluid by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel  
15 electrophoresis, dialysis, or affinity chromatography.

The monoclonal antibodies may also be made by recombinant DNA methods, such as those described in U.S. Patent No. 4,816,567. DNA encoding the monoclonal antibodies of the invention can be readily isolated and sequenced using conventional procedures (e.g.,  
20 by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells of the invention serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells  
25 such as simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. The DNA also may be modified, for example, by substituting the coding sequence for human heavy and light chain  
30 constant domains in place of the homologous murine sequences [U.S. Patent No. 4,816,567; Morrison et al., supra] or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. Such a non-immunoglobulin polypeptide can be substituted for the constant  
35 domains of an antibody of the invention, or can be substituted for

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the variable domains of one antigen-combining site of an antibody of the invention to create a chimeric bivalent antibody.

The antibodies may be monovalent antibodies. Methods for preparing monovalent antibodies are well known in the art. For example, one method involves recombinant expression of immunoglobulin light chain and modified heavy chain. The heavy chain is truncated generally at any point in the Fc region so as to prevent heavy chain crosslinking. Alternatively, the relevant cysteine residues are substituted with another amino acid residue or are deleted so as to prevent crosslinking.

In vitro methods are also suitable for preparing monovalent antibodies. Digestion of antibodies to produce fragments thereof, particularly, Fab fragments, can be accomplished using routine techniques known in the art. For instance, digestion can be performed using papain. Examples of papain digestion are described in WO 94/29348 published 12/22/94 and U.S. Patent No. 4,342,566. Papain digestion of antibodies typically produces two identical antigen binding fragments, called Fab fragments, each with a single antigen binding site, and a residual Fc fragment. Pepsin treatment yields an  $F(ab')_2$  fragment that has two antigen combining sites and is still capable of cross-linking antigen.

The Fab fragments produced in the antibody digestion also contain the constant domains of the light chain and the first constant domain ( $CH_1$ ) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain  $CH_1$  domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group.  $F(ab')_2$  antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

### 3. Humanized Antibodies

The Apo-2 antibodies of the invention may further comprise humanized antibodies or human antibodies. Humanized forms

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of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab')<sub>2</sub>, or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin [Jones et al., Nature, 321:522-525 (1986); Reichmann et al., Nature, 332:323-329 (1988); and Presta, Curr. Op. Struct. Biol., 2:593-596 (1992)].

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers [Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature, 332:323-327 (1988); Verhoeyen et al., Science, 239:1534-1536 (1988)], by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized"

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antibodies are chimeric antibodies (U.S. Patent No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important in order to reduce antigenicity. According to the "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody [Sims et al., J. Immunol., 151:2296 (1993); Chothia and Lesk, J. Mol. Biol., 196:901 (1987)]. Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies [Carter et al., Proc. Natl. Acad. Sci. USA, 89:4285 (1992); Presta et al., J. Immunol., 151:2623 (1993)].

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three dimensional models of the parental and humanized sequences. Three dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate

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immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the consensus and import sequence so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding [see, WO 94/04679 published 3 March 1994].

Transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production can be employed. For example, it has been described that the homozygous deletion of the antibody heavy chain joining region ( $J_H$ ) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge [see, e.g., Jakobovits et al., Proc. Natl. Acad. Sci. USA, 90:2551-2555 (1993); Jakobovits et al., Nature, 362:255-258 (1993); Bruggermann et al., Year in Immuno., 7:33 (1993)]. Human antibodies can also be produced in phage display libraries [Hoogenboom and Winter, J. Mol. Biol., 227:381 (1991); Marks et al., J. Mol. Biol., 222:581 (1991)]. The techniques of Cote et al. and Boerner et al. are also available for the preparation of human monoclonal antibodies (Cote et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985) and Boerner et al., J. Immunol., 147(1):86-95 (1991)].

#### 4. Bispecific Antibodies

Bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens. In the present case, one of the binding specificities is for the Apo-2, the other one is for any other antigen, and preferably for a cell-surface protein or receptor or receptor subunit.

Methods for making bispecific antibodies are known in the art. Traditionally, the recombinant production of bispecific antibodies is based on the co-expression of two immunoglobulin

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heavy-chain/light-chain pairs, where the two heavy chains have different specificities [Millstein and Cuello, Nature, 305:537-539 (1983)]. Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of ten different antibody molecules, of which only one has the correct bispecific structure. The purification of the correct molecule is usually accomplished by affinity chromatography steps. Similar procedures are disclosed in WO 93/08829, published 13 May 1993, and in Traunecker et al., EMBO J., 10:3655-3659 (1991).

According to a different and more preferred approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy-chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light-chain binding present in at least one of the fusions. DNAs encoding the immunoglobulin heavy-chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance. In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy-chain/light-chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain

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combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690 published 3 March 1994. For further details of generating bispecific antibodies see, for example, Suresh et al., Methods in Enzymology, 121:210 (1986).

#### 5. Heteroconjugate Antibodies

Heteroconjugate antibodies are also within the scope of the present invention. Heteroconjugate antibodies are composed of two covalently joined antibodies. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells [US Patent No. 4,676,980], and for treatment of HIV infection [WO 91/00360; WO 92/200373; EP 03089]. It is contemplated that the antibodies may be prepared *in vitro* using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins may be constructed using a disulfide exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptobutyrimidate and those disclosed, for example, in U.S. Pat. No. 4,676,980.

#### D. Therapeutic and Non-therapeutic Uses for Apo-2 Antibodies

The Apo-2 antibodies of the invention have therapeutic utility. Agonistic Apo-2 antibodies, for instance, may be employed to activate or stimulate apoptosis in cancer cells. Alternatively, antagonistic antibodies may be used to block excessive apoptosis (for instance in neurodegenerative disease) or to block potential autoimmune/inflammatory effects of Apo-2 resulting from NF- $\kappa$ B activation.

Apo-2 antibodies may further be used in diagnostic assays for Apo-2, e.g., detecting its expression in specific cells, tissues, or serum. Various diagnostic assay techniques known in the art may be used, such as competitive binding assays, direct or indirect sandwich assays and immunoprecipitation assays conducted in either heterogeneous or homogeneous phases [Zola, Monoclonal Antibodies: A Manual of Techniques, CRC Press, Inc. (1987) pp. 147-

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158]. The antibodies used in the diagnostic assays can be labeled with a detectable moiety. The detectable moiety should be capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety may be a radioisotope, such as  $^3\text{H}$ ,  $^{14}\text{C}$ ,  $^{32}\text{P}$ ,  $^{35}\text{S}$ , or  $^{125}\text{I}$ , a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin, or an enzyme, such as alkaline phosphatase, beta-galactosidase or horseradish peroxidase. Any method known in the art for conjugating the antibody to the detectable moiety may be employed, including those methods described by Hunter et al., Nature, 144:945 (1962); David et al., Biochemistry, 13:1014 (1974); Pain et al., J. Immunol. Meth., 40:219 (1981); and Nygren, J. Histochem. and Cytochem., 30:407 (1982).

Apo-2 antibodies also are useful for the affinity purification of Apo-2 from recombinant cell culture or natural sources. In this process, the antibodies against Apo-2 are immobilized on a suitable support, such a Sephadex resin or filter paper, using methods well known in the art. The immobilized antibody then is contacted with a sample containing the Apo-2 to be purified, and thereafter the support is washed with a suitable solvent that will remove substantially all the material in the sample except the Apo-2, which is bound to the immobilized antibody. Finally, the support is washed with another suitable solvent that will release the Apo-2 from the antibody.

E. Kits Containing Apo-2 or Apo-2 Antibodies

In a further embodiment of the invention, there are provided articles of manufacture and kits containing Apo-2 or Apo-2 antibodies which can be used, for instance, for the therapeutic or non-therapeutic applications described above. The article of manufacture comprises a container with a label. Suitable containers include, for example, bottles, vials, and test tubes. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which includes an active agent that is effective for therapeutic or non-therapeutic applications, such as described above. The active

agent in the composition is Apo-2 or an Apo-2 antibody. The label  
on the container indicates that the composition is used for a  
specific therapy or non-therapeutic application, and may also  
indicate directions for either *in vivo* or *in vitro* use, such as  
those described above.

The kit of the invention will typically comprise the  
container described above and one or more other containers  
comprising materials desirable from a commercial and user  
standpoint, including buffers, diluents, filters, needles,  
syringes, and package inserts with instructions for use.

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The following examples are offered for illustrative  
purposes only, and are not intended to limit the scope of the  
present invention in any way.

All patent and literature references cited in the present  
specification are hereby incorporated by reference in their  
entirety.

#### EXAMPLES

All restriction enzymes referred to in the examples were  
purchased from New England Biolabs and used according to  
manufacturer's instructions. All other commercially available  
reagents referred to in the examples were used according to  
manufacturer's instructions unless otherwise indicated. The source  
of those cells identified in the following examples, and throughout  
the specification, by ATCC accession numbers is the American Type  
Culture Collection, Rockville, Maryland.

#### EXAMPLE 1

##### Isolation of cDNA clones Encoding Human Apo-2

Expressed sequence tag (EST) DNA databases (Incyte  
Pharmaceuticals, ?? do the specific databases have names that we  
must/should identify; Diane to check on what are our obligations  
per our agreement to identify them) were searched and an EST was  
identified which showed homology to the death domain of the Apo-3

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receptor [Marsters et al., Curr. Biol., 6:750 (1996)]. Human pancreas and kidney lgt10 bacteriophage cDNA libraries (both purchased from Clontech) were screened by hybridization with a synthetic oligonucleotide probe:

5 5  
GGGAGCCGCTCATGAGGAAGTTGGGCCTCATGGACAATGAGATAAAGGTGGCTAAAGCTGAGGCCA  
GCGGG 3' (SEQ ID NO:\_\_\_\_) based on the EST.

prK5 libraries were screened using techniques developed for SST project .....Avi, I've attached to the end of the appl.  
10 copies of pages from a patent application which was filed a couple of months ago on the process developed for the SST project. I'm not clear that this is what was used. it would be helpful if you could confirm (?with Austin) that this is what was used in connection with Apo-2; the info on these techniques is not yet published so I  
15 will include some of this methodology here in Ex. 1

The cDNA inserts were excised from the lambda vector arms by digestion with SalI-NotI, gel-purified, and subcloned into prK5B vector that was predigested with XhoI and NotI. The clones (3)  
20 were then sequenced in entirety. The coding regions of the cDNAs were identical except for codon 410 (using the numbering system for Fig. 1); this position encoded a leucine residue (TTG) in both pancreatic cDNAs, and a methionine residue (ATG) in the kidney cDNA, possibly due to polymorphism.

25 The entire nucleotide sequence of Apo-2 is shown in Figure 1 (SEQ ID NO:\_\_\_\_). Clone 27868 (also referred to as Apo-2 clone \_\_\_\_ deposited as ATCC \_\_\_\_\_, as indicated below) contains a single open reading frame with an apparent translational initiation site at nucleotide positions 140-142 and ending at the stop codon  
30 found at nucleotide positions 1373-1375 (Fig. 1; SEQ ID NO:\_\_\_\_) [Kozak et al., supra]. The predicted polypeptide precursor is 411 amino acids long, a type I transmembrane protein, and has a calculated molecular weight of approximately 45 kDa. Hydropathy analysis (not shown) suggested the presence of a signal sequence  
35 (residues 1-48), followed by an extracellular domain (residues 49-

182), a transmembrane domain (residues 183-208), and an intracellular domain (residues 209-411) (Fig. 2A; SEQ ID NO:\_\_\_).

TNF receptor family proteins are typically characterized by the presence of multiple (usually four) cysteine-rich domains in their extracellular regions -- each cysteine-rich domain being approximately 45 amino acids long and containing approximately 6, regularly spaced, cysteine residues. Based on the crystal structure of the type 1 TNF receptor, the cysteines in each domain typically form three disulfide bonds in which usually cysteines 1 and 2, 3 and 5, and 4 and 6 are paired together. Like DR4, Apo-2 contains two extracellular cysteine-rich pseudorepeats (Fig. 2A), whereas other identified mammalian TNFR family members contain three or more such domains [Smith et al., Cell, 76:959 (1994)].

The cytoplasmic region of Apo-2 contains a death domain (amino acid residues 324-391 shown in Fig. 1; see also Fig. 2A) which shows significantly more amino acid sequence identity to the death domain of DR4 (64%) than to the death domain of TNFR1 (30%); CD95 (19%); or Apo-3/DR3 (29%) (Fig. 2B). Four out of six death domain amino acids that are required for signaling by TNFR1 [Tartaglia et al., supra] are conserved in Apo-2 while the other two residues are semi-conserved (see Fig. 2B).

Based on an alignment analysis (using the ALIGN™ computer program) of the full-length sequence, Apo-2 shows more sequence identity to DR4 (55%) than to other apoptosis-linked receptors, such as TNFR1 (19%); CD95 (17%); or Apo-3 (also referred to as DR3, WSL-1 or TRAMP) (29%).

## EXAMPLE 2

### A. Expression of Apo-2 ECD

A soluble extracellular domain (ECD) fusion construct was prepared. The Apo-2 ECD (amino acid residues 1-184 shown in Figure 1) was obtained by PCR and fused to a C-terminal Flag epitope tag (Sigma). The Flag epitope-tagged molecule was then inserted into pRK5, and expressed by transient transfection into human 293 cells (ATCC CRL 1573).

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After a 48 hour incubation, the cell supernatants were collected and either used directly for co-precipitation studies (see Example 3) or subjected to purification of the Apo-2 ECD-Flag by affinity chromatography on anti-Flag agarose beads, according to manufacturer's instructions (Sigma).

B. Expression of Apo-2 ECD as an Immunoadhesin

A soluble Apo-2 ECD immunoadhesin construct was prepared. The Apo-2 ECD (amino acids 1-184 shown in Fig. 1) was fused to the hinge and Fc region of human immunoglobulin G<sub>1</sub> heavy chain in pRK5 as described previously [Ashkenazi et al., Proc. Natl. Acad. Sci., 88:10535-10539 (1991)]. The immunoadhesin was expressed by transient transfection into human 293 cells and purified from cell supernatants by protein A affinity chromatography, as described by Ashkenazi et al., supra.

EXAMPLE 3

Immunoprecipitation Assay Showing Binding Interaction Between Apo-2 and Apo-2 Ligand

To determine whether Apo-2 and Apo-2L interact or associate with each other, supernatants from mock-transfected 293 cells or from 293 cells transfected with Apo-2 ECD-Flag (described in Example 2 above) (5 ml) were incubated with 5 µg poly-histidine-tagged soluble Apo-2L [Pitti et al., supra] for 30 minutes at room temperature and then analyzed for complex formation by a co-precipitation assay.

The samples were subjected to immunoprecipitation using 25 µl anti-Flag conjugated agarose beads (Sigma) or Nickel-conjugated agarose beads (Qiagen). After a 1.5 hour incubation at 4 C, the beads were spun down and washed four times in phosphate buffered saline (PBS). Apo-2L was precipitated through the Flag-tagged Apo-2 ECD; by using Nickel-agarose, the Apo-2 ECD precipitated through the His-tagged Apo-2L. The precipitated proteins were released by boiling the beads for 5 minutes in SDS-PAGE buffer, resolved by electrophoresis on 12% polyacrylamide

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gels, and then detected by immunoblot with anti-Apo-2L or anti-Flag antibody (2  $\mu$ g/ml) as described in Marsters et al., J. Biol. Chem., in press (1997).

The results, shown in Figure 2A, indicate that the Apo-2 ECD and Apo-2L can associate with each other.

The binding interaction was further analyzed by purifying Apo-2 ECD from the transfected 293 cell supernatants with anti-Flag beads (see Example 2) and then analyzing the samples on a BIAcore™ instrument. The BIAcore™ analysis indicated a dissociation constant ( $K_d$ ) of about 1 nM. BIAcore™ analysis also showed that the Apo-2 ECD is not capable of binding other apoptosis-inducing TNF family members, namely, TNF-alpha (Genentech, Inc., Pennica et al., Nature, 312:712 (1984), lymphotoxin-alpha (Genentech, Inc.), or Fas/Apo-1 ligand (Alexis Biochemicals). The data thus shows that Apo-2 is a specific receptor for Apo-2L.

#### EXAMPLE 4

##### Induction of Apoptosis by Apo-2

Because death domains can function as oligomerization interfaces, over-expression of receptors that contain death domains may lead to activation of signaling in the absence of ligand [Frazer et al., supra, Nagata et al., supra]. To determine whether Apo-2 was capable of inducing cell death, human 293 cells or HeLa cells (ATCC CCL 2.2) were transiently transfected by calcium phosphate precipitation (293 cells) or electroporation (HeLa cells) with a pRK5 vector or pRK5-based plasmids encoding Apo-2 and/or CrmA. When applicable, the total amount of plasmid DNA was adjusted by adding vector DNA. Apoptosis was assessed 24 hours after transfection by morphology (Fig. 4A); DNA fragmentation (Fig. 4B); or by FACS analysis of phosphatidylserine exposure (Fig. 4C) as described in Marsters et al., Curr. Biol., 6:1669 (1996). As shown in Figs. 4A and 4B, the Apo-2 transfected 293 cells underwent marked apoptosis.

For samples assayed by FACS, the HeLa cells were co-transfected with pRK5-CD4 as a marker for transfection and

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apoptosis was determined in CD4-expressing cells; FADD was co-transfected with the Apo-2 plasmid; the data are means  $\pm$  SEM of at least three experiments, as described in Marsters et al., Curr. Biol., 6:1669 (1996). The caspase inhibitors, DEVD-fmk (Enzyme Systems) or z-VAD-fmk (Research Biochemicals Intl.) were added at 200  $\mu$ M at the time of transfection. As shown in Fig. 4C, the caspase inhibitors CrmA, DEVD-fmk, and z-VAD-fmk blocked apoptosis induction by Apo-2, indicating the involvement of Ced-3-like proteases in this response.

FADD is an adaptor protein that mediates apoptosis activation by CD95, TNFR1, and Apo-3/DR3 [Nagata et al., supra], but does not appear necessary for apoptosis induction by Apo-2L [Marsters et al., supra] or by DR4 [Pan et al., supra]. A dominant-negative mutant form of FADD, which blocks apoptosis induction by CD95, TNFR1, or Apo-3/DR3 [Frazer et al., supra; Nagata et al., supra; Chinnayian et al., supra] did not inhibit apoptosis induction by Apo-2 when co-transfected into HeLa cells with Apo-2 (Fig. 4C). These results suggest that Apo-2 signals apoptosis independently of FADD. Consistent with this conclusion, a glutathione-S-transferase fusion protein containing the Apo-2 cytoplasmic region did not bind to *in vitro* transcribed and translated FADD (data not shown).

#### EXAMPLE 5

##### Inhibition of Apo-2L Activity by Soluble Apo-2 ECD

Soluble Apo-2L (0.5  $\mu$ g/ml, prepared as described in Pitti et al., supra) was pre-incubated for 1 hour at room temperature with PBS buffer or affinity-purified Apo-2 ECD (5  $\mu$ g/ml) together with anti-Flag antibody (Sigma) (1  $\mu$ g/ml) and added to HeLa cells. After a 5 hour incubation, the cells were analyzed for apoptosis by FACS (as above) (Fig. 4D).

Apo-2L induced marked apoptosis in HeLa cells, and the soluble Apo-2 ECD was capable of blocking Apo-2L action (Fig. 4D), confirming a specific interaction between Apo-2L and Apo-2. Similar results were obtained with the Apo-2 ECD immunoadhesin



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shown in Fig. 5B. The Apo-2L induced a significant NF- $\kappa$ B activation in the treated HeLa cells but not in the treated MCF7 cells; the TNF-alpha induced a more pronounced activation in both cell lines. Several studies have disclosed that NF- $\kappa$ B activation by TNF can protect cells against TNF-induced apoptosis [Nagata, supra].

The effects of a NF- $\kappa$ B inhibitor, ALLN (N-acetyl-Leu-Leu-norleucinal) and a transcription inhibitor, cyclohexamide, were also tested. The HeLa cells (placed in 6-well dishes) were preincubated with PBS buffer, ALLN (Calbiochem) (40  $\mu$ g/ml) or cyclohexamide (Sigma) (50  $\mu$ g/ml) for 1 hour before addition of Apo-2L (1  $\mu$ g/ml). After a 5 hour incubation, apoptosis was analyzed by FACS (see Fig. 5C).

The results are shown in Fig. 5C. Both ALLN and cyclohexamide increased the level of Apo-2L-induced apoptosis in the HeLa cells. The data indicates that Apo-2L can induce protective NF- $\kappa$ B-dependent genes. The data also indicates that Apo-2L is capable of activating NF- $\kappa$ B in certain cell lines and that both Apo-2 and DR4 may mediate that function.

#### EXAMPLE 7

##### Northern Blot Analysis

Expression of Apo-2 mRNA in human tissues was examined by Northern blot analysis. Human RNA blots were hybridized to a 4.6 kilobase  $^{32}$ P-labelled DNA probe based on the full length Apo-2 cDNA; the probe was generated by digesting the pRK5-Apo-2 plasmid with EcoRI. Human fetal RNA blot MTN (Clontech) and human adult RNA blot MTN-II (Clontech) were incubated with the DNA probes. Blots were incubated with the probes in hybridization buffer (5X SSPE; 2X Denhardt's solution; 100 mg/mL denatured sheared salmon sperm DNA; 50% formamide; 2% SDS) for 60 hours at 42°C. The blots were washed several times in 2X SSC; 0.05% SDS for 1 hour at room temperature, followed by a 30 minute wash in 0.1X SSC; 0.1% SDS at 50°C. The blots were developed after overnight exposure.

As shown in Fig. 6, a predominant mRNA transcript of

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approximately 4.6kb was detected in multiple tissues. Expression was relatively high in fetal and adult liver and lung, and in adult ovary and peripheral blood leukocytes (PBL), while no mRNA expression was detected in fetal and adult brain. Intermediate levels of expression were seen in adult colon, small intestine, testis, prostate, thymus, pancreas, kidney, skeletal muscle, placenta, and heart. . . Several adult tissues that express Apo-2, e.g., PBL, ovary, and spleen, have been shown previously to express DR4 [Pan et al., supra], however, the relative levels of expression of each receptor mRNA appear to be different.

EXAMPLE 8

Chromosomal Localization of the Apo-2 gene

Chromosomal localization of the human Apo-2 gene was examined by radiation hybrid (RH) panel analysis. RH mapping was performed by PCR using a human-mouse cell radiation hybrid panel (Research Genetics) and primers based on the coding region of the Apo-2 cDNA [Gelb et al., Hum. Genet., 98:141 (1996)]. Analysis of the PCR data using the Stanford Human Genome Center Database indicates that Apo-2 is linked to the marker D8S481, with an LOD of 11.05; D8S481 is linked in turn to D8S2055, which maps to human chromosome 8p21.2.

To Applicants present knowledge, to date, no other member of the TNFR gene family has been located to chromosome 8.

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\* \* \* \* \*

Deposit of Material

The following materials have been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, USA (ATCC):

<u>Material</u>	<u>ATCC Dep. No.</u>	<u>Deposit Date</u>
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This deposit was made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of a viable culture of the deposit for 30 years from the date of deposit. The deposit will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Genentech, Inc. and ATCC, which assures permanent and unrestricted availability of the progeny of the culture of the deposit to the public upon issuance of the pertinent U.S. patent or upon laying open to the public of any U.S. or foreign patent application, whichever comes first, and assures availability of the progeny to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC §122 and the Commissioner's rules pursuant thereto (including 37 CFR §1.14 with particular reference to 886 OG 638).

The assignee of the present application has agreed that if a culture of the materials on deposit should die or be lost or destroyed when cultivated under suitable conditions, the materials will be promptly replaced on notification with another of the same. Availability of the deposited material is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the

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invention. The present invention is not to be limited in scope by  
the construct deposited, since the deposited embodiment is intended  
as a single illustration of certain aspects of the invention and  
any constructs that are functionally equivalent are within the  
scope of this invention. The deposit of material herein does not  
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illustrations that it represents. Indeed, various modifications of  
the invention in addition to those shown and described herein will  
become apparent to those skilled in the art from the foregoing  
description and fall within the scope of the appended claims.

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## **A Second Cell Death Receptor for the Cytokine Apo-2 Ligand**

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The cytokine Apo-2 ligand (Apo-2L/TRAIL) belongs to the tumor necrosis factor family and activates apoptosis in tumor cells. Recently, a receptor for Apo-2L called DR4 was identified. Here we describe a second receptor for Apo-2L, called Apo-2. The Apo-2 protein is 55 % identical to DR4. Apo-2 mRNA is expressed in multiple human tissues, some of which also express DR4. The soluble extracellular domain of Apo-2 binds Apo-2L, and inhibits Apo-2L function. Like DR4, Apo-2 contains a cytoplasmic "death domain", and activates FADD-independent, caspase-dependent apoptosis. Both Apo-2 and DR4 also activate nuclear factor- $\kappa$ B. Hence, Apo-2L can signal similar cellular responses via two distinct receptors.

Apoptosis (programmed cell death) plays a critical role in development and homeostasis of metazoans (1). The cell death program has three essential types of elements: activators, inhibitors, and effectors; in *C. elegans*, these components are encoded respectively by the *Ced-4*, *Ced-9*, and *Ced-3* genes. Metazoan cells contain a latent apoptotic program, which can be activated by specific cues from inside or outside the cell. Fas/Apo-1 ligand (CD95L) and tumor necrosis factor (TNF) are important extracellular activators of apoptosis in the mammalian immune system; their cognate receptors, CD95 and TNFR1, contain cytoplasmic death domains (2, 3). The death domains of TNFR1 and CD95 activate the cell's apoptotic machinery through interaction with the death domains of the adaptor proteins FADD (also called MORT1) (4, 5) and TRADD (6, 7). Upon activation by ligand, CD95 recruits FADD directly, while TNFR1 binds FADD indirectly, via TRADD. FADD in turn activates the *Ced-3*-related protease MACH $\alpha$ /FLICE (caspase 8), thereby initiating a series of caspase-dependent events that lead to cell death (8, 9).

The cytokine Apo-2L (10) (also called TRAIL) (11) is structurally related to CD95L and TNF; Apo-2L activates rapid apoptosis in tumor cell lines, acting independently of CD95 and TNFR1 (10). Recently, a receptor for Apo-2L designated DR4 was described

(12). DR4 belongs to the TNFR gene family, contains a cytoplasmic death domain, and activates apoptosis. The extracellular domain (ECD) of DR4, expressed as a soluble immunoglobulin-fusion protein (immunoadhesin), binds to Apo-2L and blocks Apo-2L-induced cell death, indicating that DR4 is a specific receptor for Apo-2L (12).

By searching expressed sequence tag (EST) DNA databases, we identified an EST that showed homology to death domains. Based on this EST, we isolated cDNAs from human pancreas and kidney encoding an undescribed member of the TNFR family (Fig. 1). We named this protein Apo-2 (see below). The predicted Apo-2 precursor is a 411 amino acid type I transmembrane protein, with a calculated molecular weight of ~45 kDa. Overall, Apo-2 shows more sequence identity to DR4 (55 %) than to other apoptosis-linked receptors, namely, Apo-3 (also called DR3, WSL-1, or TRAMP) (13-16) (29 %), TNFR1 (19 %), or CD95 (17 %). Like DR4, Apo-2 contains only two extracellular cysteine-rich pseudo-repeats (Fig. 1A), whereas other mammalian TNFR family members contain three or more such domains (17). The cytoplasmic region of Apo-2 contains a death domain which shows significantly more identity to the death domain of DR4 (64 %) than to the death domain of Apo-3/DR3 (29 %), TNFR1 (30 %), or CD95 (19 %). Notably, four out of six death domain amino acids that are required for signaling by TNFR1 (18) are conserved in Apo-2, while the other two are semiconserved (Fig. 1B).

To determine the pattern of Apo-2 mRNA expression, we analyzed a panel of human tissues by Northern hybridization (Fig. 1C). We detected a single Apo-2 mRNA transcript of ~4.6 kb in multiple tissues. Expression was relatively high in fetal and adult liver and lung, and in adult ovary and peripheral blood leukocytes (PBL), while no mRNA expression was detected in fetal and adult brain. Several adult tissues that express Apo-2 (*e.g.*, PBL, ovary, and spleen) have been shown to express DR4 (12).

We investigated the chromosomal location of the human Apo-2 gene by radiation hybrid analysis (not shown). *Apo-2* mapped to chromosome 8p21.2 (19, 20). To date, no other member of the TNFR gene family has been mapped to chromosome 8 (21).

The relatively high sequence homology between Apo-2 and DR4 suggested that the two receptors may interact with a common ligand. To test this notion, we expressed the Apo-2 ECD as a soluble, Flag-epitope-tagged protein in human 293 cells. We incubated the ECD with poly-histidine-tagged soluble Apo-2L (10), and analyzed complex formation by a co-precipitation assay (Fig. 2). By using anti-Flag antibody-agarose, we were able to precipitate Apo-2L through the Flag-tagged Apo-2 ECD; similarly, by using nickel-agarose, we were able to precipitate the Apo-2 ECD through the His-tagged Apo-2L. These results indicate that the Apo-2 ECD and Apo-2L can associate. BIACORE analysis of the binding interaction indicated a dissociation constant ( $K_D$ ) of  $\sim 1$  nM; furthermore, this analysis showed that the Apo-2 ECD does not bind to other cytotoxic TNF family members, namely, TNF, lymphotoxin- $\alpha$  (LT- $\alpha$ ), or CD95L (data not shown). Hence, Apo-2 is a specific receptor for Apo-2L.

Because death domains function as oligomerization interfaces, overexpression of receptors that contain such domains leads to activation of signaling in the absence of ligand (2, 3). To investigate whether Apo-2 is capable of inducing cell death, we transfected human 293 or HeLa cells transiently by an Apo-2 expression plasmid, and assessed the level of apoptosis after 24 hours. Apo-2-transfected cells underwent marked apoptosis, as indicated by morphological changes, internucleosomal DNA fragmentation, and exposure of phosphatidylserine on the cell surface (Fig. 3 A-C). The caspase inhibitors CrmA, DEVD-fmk, and z-VAD-fmk blocked apoptosis activation by Apo-2, indicating the involvement of *Ced-3* -like proteases in this response. Consistent with previous work (22), Apo-2L induced marked apoptosis in HeLa cells. The activity of Apo-2L was blocked by the soluble Apo-2 ECD, as well as by immunoadhesins based on the Apo-2 ECD or the DR4 ECD, but not the ECD of TNFR1 (Fig. 3 D). Titration of the Apo-2 immunoadhesin showed half-maximal inhibition of Apo-2L activity at concentration of  $\sim 0.3$  nM (Fig. 3E), confirming a specific, high affinity interaction between Apo-2L and Apo-2.

The adaptor protein FADD mediates apoptosis activation by CD95, TNFR1, and Apo-3/DR3 (3). Deletion of the N-terminal 80 amino acids of FADD creates a dominant-negative mutant (FADD-DN) that blocks apoptosis induction by CD95, TNFR1, or Apo-3/DR3 (7, 14, 23), but not by Apo-2L (22) or DR4 (12). FADD-DN did not inhibit apoptosis induction by Apo-2 (Fig. 3C), indicating that Apo-2 signals apoptosis independently of FADD. Consistent with this conclusion, a glutathione-S-transferase fusion protein containing the Apo-2 cytoplasmic region did not bind to *in vitro* transcribed and translated FADD (data not shown).

In addition to inducing apoptosis, TNFR1, CD95, and Apo-3/DR3 activate the transcription factor NF- $\kappa$ B (13-16, 18, 24), which controls the expression of multiple immunomodulatory genes (25). Previous work suggested that DR4 is not linked to NF- $\kappa$ B, because transfection of DR4 in MCF7 cells did not lead to NF- $\kappa$ B activation (12). However, upon transfection into HeLa cells, both Apo-2 and DR4 induced significant NF- $\kappa$ B activation as measured by electrophoretic mobility shift assay (Fig. 4A); the level of activation was comparable to activation by Apo-3/DR3. Antibody to the p65/RelA subunit of NF- $\kappa$ B inhibited the mobility of the NF- $\kappa$ B probe, implicating p65 in the response to all three receptors. We tested also whether Apo-2L itself regulates NF- $\kappa$ B activity (Fig. 4B). Apo-2L induced a detectable NF- $\kappa$ B activation in HeLa cells, but not in MCF7 cells; TNF induced a more pronounced activation in both cell lines (Fig. 4B). Several studies have shown that NF- $\kappa$ B activation by TNF protects cells against TNF-induced apoptosis (3). The NF- $\kappa$ B inhibitor ALLN (N-acetyl-Leu-Leu-norleucinal) and the transcription inhibitor cyclohexamide each increased the level of Apo-2L-induced apoptosis in HeLa cells (Fig. 4C), suggesting that Apo-2L is capable of inducing expression of anti-apoptotic genes through NF- $\kappa$ B. These data indicate that Apo-2L activates NF- $\kappa$ B in a cell type dependent manner, and that both Apo-2 and DR4 can mediate this function. Dose-response analysis in several cell types showed that TNF activates NF- $\kappa$ B at 100-1000 fold lower

concentrations than Apo-2L (S.A.M. and A.A., unpublished data), suggesting TNF and Apo-2L may activate this transcription factor through different mechanisms.

The TNF ligand family contains about a dozen members; each is believed to interact with a single receptor, except TNF and LT- $\alpha$  (21). TNF and LT- $\alpha$  both interact with the same two receptors, TNFR1 and TNFR2. The identification of Apo-2 demonstrates that Apo-2L is another TNF family member that interacts with more than one receptor. Unlike TNFR1 and TNFR2, which are unrelated in their cytoplasmic region, DR4 and Apo-2 both have cytoplasmic death domains. Moreover, while TNFR1 and TNFR2 differ in their signaling functions, DR4 and Apo-2 appear to be similar in function, because they both regulate apoptosis as well as NF- $\kappa$ B. DR4 and Apo-2 share relatively high death domain sequence homology; further, death signaling by these receptors is independent of FADD but dependent on caspases. Thus, DR4 and Apo-2 may use common signaling elements that differ from those used by TNFR1, CD95 and Apo-3/DR3 to activate the cell's apoptotic machinery.

It is intriguing that Apo-2L uses two distinct receptors to stimulate a similar set of cellular responses. The physiological function of Apo-2L is ~~unknown~~ <sup>not fully understood</sup>, although there is evidence that Apo-2L may play a role in early elimination of peripheral blood T cells after stimulation by interleukin-2 (22). The Apo-2L mRNA is expressed in multiple human tissues (10, 11), as are the mRNAs of Apo-2 and DR4 (12). While Apo-2L is cytotoxic to a broad spectrum of tumor cell lines, it is unlikely that the tissues that express Apo-2 and/or DR4 are constitutively sensitive to Apo-2L-induced apoptosis. Consistent with this latter notion, Apo-2L does not induce any obvious toxicity after injection into mice, nor does it induce apoptosis in primary umbilical vein endothelial cells (J.P.S. and A.A., unpublished results). Hence, cellular sensitivity to induction of cell death by Apo-2L probably is regulated by additional mechanisms besides control of Apo-2 and DR4 expression. These mechanisms may include release of soluble receptors, NF- $\kappa$ B-dependent expression of protective genes, or downregulation of components in the Apo-2L

death signaling pathway. The broad sensitivity of tumor cell lines to Apo-2L-induced cell death suggests that neoplastic transformation perhaps sensitizes cells to the apoptotic effect of this cytokine.



## Figure Legends

**Fig. 1.** Primary structure and mRNA expression of Apo-2. (A) Deduced amino acid sequence of human Apo-2. The nucleotide sequence is available through Genbank accession number #. The predicted signal peptide (underlined), transmembrane domain (boxed), and death domain (dashed underline) are shown. The cysteines of the two cysteine-rich domains are individually underlined. By screening human cDNA libraries (Clontech) with a hybridization probe based on a death-domain-related EST, we isolated three cDNA clones encoding Apo-2: two from pancreas and one from kidney. The overlapping coding region of the cDNAs was identical except for codon 410; this position encodes a leucine (TTG) in both pancreatic cDNAs, and a methionine (ATG) in the kidney cDNA. The signal peptide cleavage site was determined by N-terminal amino acid sequence analysis of the soluble Apo-2 ECD expressed in 293 cells. Abbreviations for the amino acids are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr. (B) Comparison of the death domain sequences of human Apo-2, DR4, Apo-3/DR3, TNFR1, and Fas/Apo1 (CD95). Asterisks indicate residues that are essential for death signaling by TNFR1 (18). (C) Expression of Apo-2 mRNA in fetal and adult human tissues was analyzed by Northern hybridization of tissue poly A RNA blots (Clontech) with a probe based on the full-length Apo-2 cDNA. PBL, peripheral blood leukocytes; sm. intest., small intestine; sk. muscle, skeletal muscle.

**Fig. 2** Interaction of the Apo-2 ECD with Apo-2L. The Apo-2 ECD (amino acids 1-184) was fused to a C-terminal Flag epitope tag (Sigma), inserted into pRK5, and expressed by transient transfection in 293 cells. Supernatants from mock-transfected cells or from cells transfected by Apo-2ECD-Flag (5 ml) were incubated with 5 µg soluble, poly-His-tagged Apo-2L (10) for 30 min at 24°C and subjected to immunoprecipitation (IP) with anti-Flag-conjugated (Sigma) or Nickel-conjugated (Qiagen) agarose beads. The precipitated

proteins were resolved by electrophoresis on 12 % polyacrylamide gels, and detected by Western blot (WB) with anti-Apo-2L or anti-Flag antibody as described (26).

**Fig. 3** Induction of apoptosis by Apo-2 and inhibition of Apo-2L activity by soluble Apo-2 ECD. Human 293 cells (**A, B**) or HeLa cells (**C**) were transiently transfected by pRK5-based plasmids encoding Apo-2, CrmA, and/or FADD residues 80-205 (FADD-DN) as indicated. Apoptosis was assessed 24 hours after transfection by morphology (**A**), by DNA fragmentation (**B**), or by FACS analysis of phosphatidylserine exposure (**C**), as described (13). Transfections were done by calcium phosphate precipitation for 293 cells and by electroporation for HeLa cells; 8  $\mu$ g of each plasmid was added per transfection, and where applicable, the total amount of plasmid DNA was equalized with vector DNA. In (**C**), cells were co-transfected in addition with pRK5-CD4 (4  $\mu$ g) as a marker for transfection and apoptosis was determined in CD4-expressing cells. The caspase inhibitors DEVD-fmk (Enzyme Systems) or z-VAD-fmk (Rersearch Biochemicals International) were added at 200  $\mu$ M at the time of transfection. (**D, E**) Apo-2L (0.5  $\mu$ g/ml) was preincubated for 1 hr at 24°C with buffer, or with affinity-purified Flag-tagged Apo-2 ECD (5  $\mu$ g/ml) together with anti-Flag antibody (1  $\mu$ g/ml), or with each indicated receptor immunoadhesin (20  $\mu$ g/ml or a range of doses in panel E), and added to HeLa cells. After a 5 hr incubation, the cells were analyzed for apoptosis by FACS as above. Immunoadhesins were generated by fusing each receptor ECD to the hinge and Fc regions of human IgG1 as described (27). The data in panel C and D are means  $\pm$  SEM of at least 3 experiments.

**Fig. 4** Activation of NF- $\kappa$ B by Apo-2, DR4, and Apo-2L. (**A**) HeLa cells were transfected with expression plasmids encoding the indicated proteins. Nuclear extracts were prepared 24 hr later and analyzed by NF- $\kappa$ B-specific electrophoretic mobility shift assay as described (26). Extracts were incubated with  $^{32}$ P-labelled NF- $\kappa$ B-specific oligonucleotide probe alone, or together with 50-fold excess of unlabelled probe, or in the

presence of anti-p65/RelA antibody (Santa Cruz Biotechnology) as indicated. (B) HeLa or MCF7 cells were treated with buffer, Apo-2L, or TNF (1 $\mu$ g/ml) and assayed for NF- $\kappa$ B activity as above. (C) HeLa cells were preincubated with vehicle, ALLN (Calbiochem) (40  $\mu$ g/ml), or cyclohexamide (sigma) (50  $\mu$ g/ml) for 1 hr before addition of Apo-2L (0.25  $\mu$ g/ml). Five hr later, apoptosis was analyzed by FACS as in Fig. 3C. Data are means $\pm$  SEM of triplicates.

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28. We thank C. Clark for comments on the manuscript, D. V. Goeddel for CrmA and FADD-DN expression plasmids, V. M. Dixit for DR4 and DR4-IgG expression plasmids, P. Juhani, P. Ng, M. Vasser for DNA synthesis, M. Hamner for DNA sequencing help, and K. D. Bauer for advice on FACS.

**A**

```

1  MEORGONAPAAAGARKRHGPGPREARGARPGRLVPKTLVLVVAALLVSAESALITQOD
61  LAPQORAAPOQKRSPSEGLCPGHHISEDGRDCISCKYGODYSTHWNDLLFLRCRTRCD
121  SGEVELSPCTTTRNTVCQCEEGTFREEDSPENCRKCRGTGCPRGMVKVGDCPTPWSDIECVH
181  KESGIIIGVTVAAVLIVAVFVCKSLMKKVLPLYKGCISGGGGDPERVDRSSORRLLVPA
241  NVLNEIVSILQPTQVPEQEMEVOEPAEPTGVNMLSPGESEHLLLEPAEAERSQRRRLVPA
301  NEGDPTELTRQCFFDADLVFPDSWEPLMRKLGIMDNEIKVAKAEAA--GHRD*TL
361  VNKTGRDASVHTLLDALETLGERLAKQKIEDHLLSSGKFMYLEGNADSALS

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**B**

Apo2	FADLVPEFDSWEP	L	M	R	K	I	G	L	M	D	N	E	I	K	V	A	K	A	E	A	-	-	G	H	R	D	T	L	
DR4	FANIVPEFDSWD	Q	L	M	R	Q	I	D	L	T	K	N	E	I	D	V	V	R	A	G	T	A	-	-	G	P	G	D	A
Apo3/DR3	VMDAVPARRWKE	F	V	R	T	L	G	L	R	E	A	E	I	E	A	V	E	V	E	I	G	R	-	-	F	R	D	Q	
TNFR1	VVENVPPLRWKE	F	V	R	R	I	G	L	S	D	H	E	I	D	R	L	E	L	O	N	G	R	-	C	L	R	E		
Fas/Apo1	IAGVMTLSQVKG	F	V	R	K	N	G	V	N	E	A	K	I	D	E	I	K	N	D	N	V	Q	D	T	A	E	Q		

Apo2	YTMLIKWV	N	K	T	G	R	D	-	A	S	V	H	T	L	L	D	A	L	E	T	L	G	E	R	L	A	K	Q
DR4	YAMLKWKV	N	K	T	G	R	N	-	A	S	I	H	T	L	L	D	A	L	E	R	M	E	E	R	H	A	K	
Apo3/DR3	YEMLKRRW	R	Q	Q	P	-	-	-	A	G	L	G	A	V	A	A	L	E	R	M	G	I	D	G	C	V	E	
TNFR1	YSMLATW	R	R	R	T	P	R	R	E	A	T	L	E	L	L	G	R	V	L	R	D	M	L	L	G	C		
Fas/Apo1	-QLLRN	W	H	Q	L	H	G	K	K	E	A	Y	-	D	T	L	I	K	D	L	K	K	A	N	L	C		

**C**

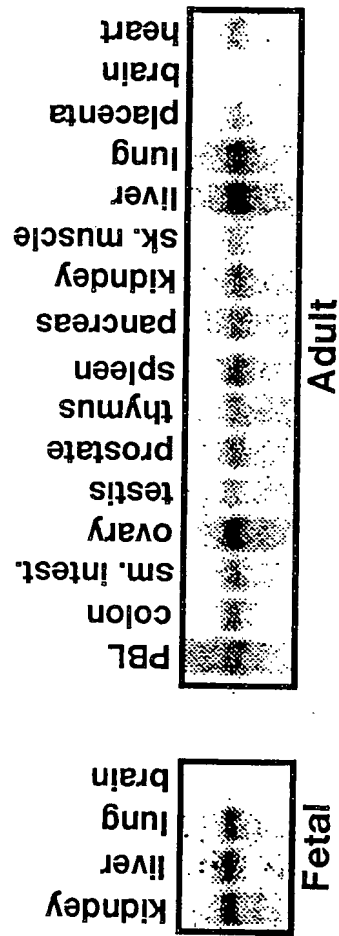


Figure 1  
Sheridan et al.

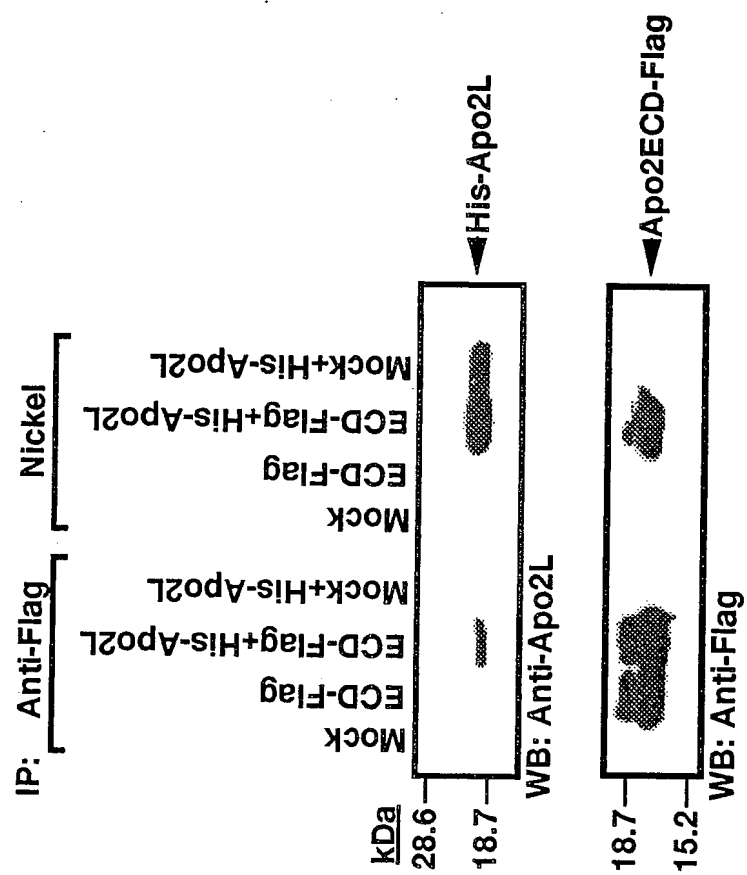


Figure 2  
Sheridan et al.

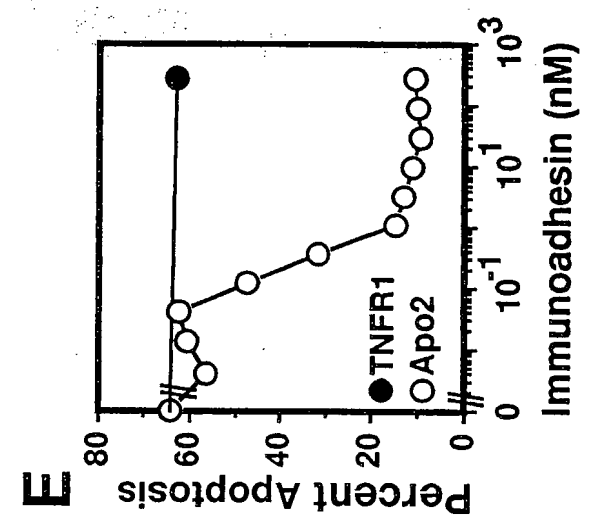
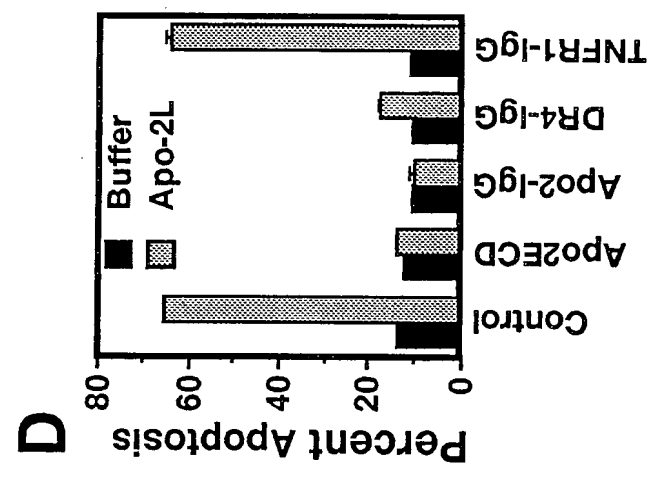
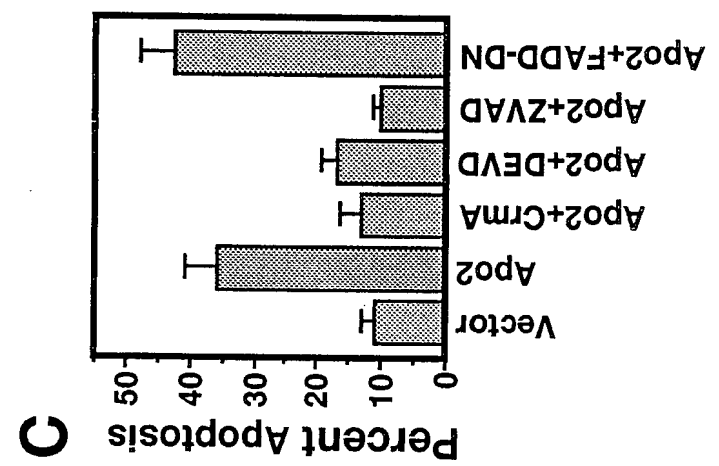
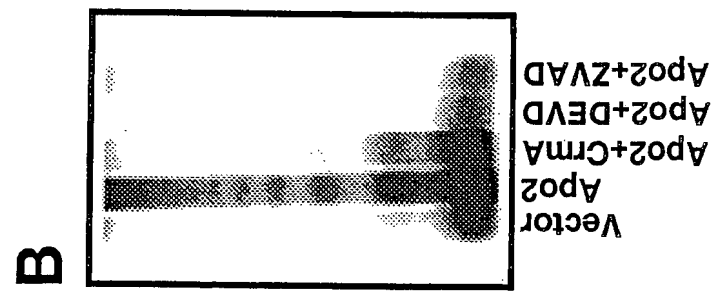
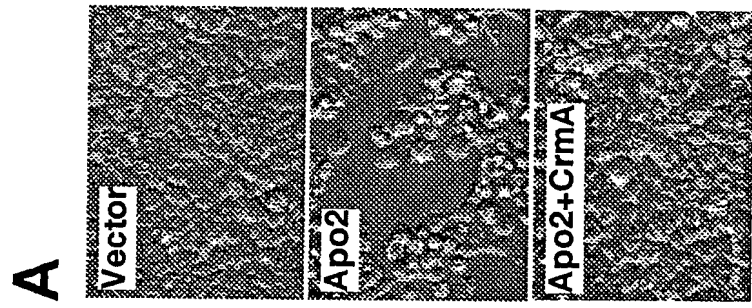


Figure 3  
Sheridan et al.



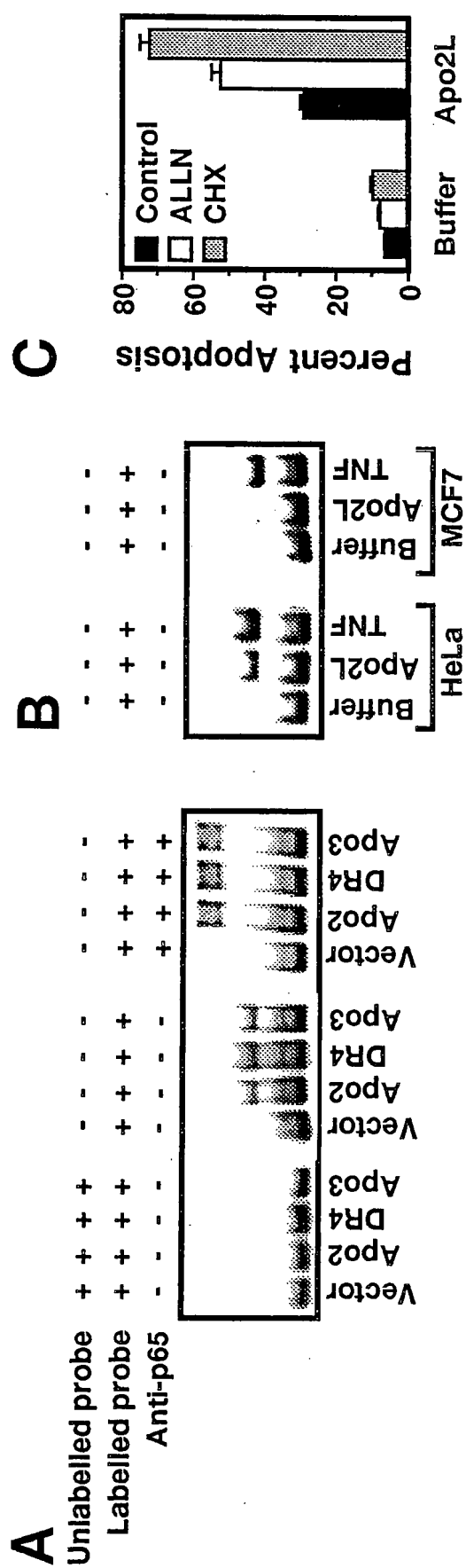


Figure 4  
Sheridan et al.

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Draft #2 - May 13, 1997

Apo-2 Receptor

FIELD OF THE INVENTION

The present invention relates generally to the identification, isolation, and recombinant production of novel polypeptides, designated herein as "Apo-2".

BACKGROUND OF THE INVENTION

Apoptosis or "Programmed Cell Death"

Control of cell numbers in mammals is believed to be determined, in part, by a balance between cell proliferation and cell death. One form of cell death, sometimes referred to as necrotic cell death, is typically characterized as a pathologic form of cell death resulting from some trauma or cellular injury. In contrast, there is another, "physiologic" form of cell death which usually proceeds in an orderly or controlled manner. This orderly or controlled form of cell death is often referred to as "apoptosis" [see, e.g., Barr et al., Bio/Technology, 12:487-493 (1994); Steller et al., Science, 267:1445-1449 (1995)]. Apoptotic cell death naturally occurs in many physiological processes, including embryonic development and clonal selection in the immune system [Itoh et al., Cell, 66:233-243 (1991)]. Decreased levels of apoptotic cell death have been associated with a variety of pathological conditions, including cancer, lupus, and herpes virus infection [Thompson, Science, 267:1456-1462 (1995)]. Increased levels of apoptotic cell death may be associated with a variety of other pathological conditions, including AIDS, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, multiple sclerosis, retinitis pigmentosa, cerebellar degeneration, aplastic

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anemia, myocardial infarction, stroke, reperfusion injury, and toxin-induced liver disease [see, Thompson, supra].

Apoptotic cell death is typically accompanied by one or more characteristic morphological and biochemical changes in cells, such as condensation of cytoplasm, loss of plasma membrane microvilli, segmentation of the nucleus, degradation of chromosomal DNA or loss of mitochondrial function. A variety of extrinsic and intrinsic signals are believed to trigger or induce such morphological and biochemical cellular changes [Raff, Nature, 356:397-400 (1992); Steller, supra; Sachs et al., Blood, 82:15 (1993)]. For instance, they can be triggered by hormonal stimuli, such as glucocorticoid hormones for immature thymocytes, as well as withdrawal of certain growth factors [Watanabe-Fukunaga et al., Nature, 356:314-317 (1992)]. Also, some identified oncogenes such as *myc*, *rel*, and *E1A*, and tumor suppressors, like *p53*, have been reported to have a role in inducing apoptosis. Certain chemotherapy drugs and some forms of radiation have likewise been observed to have apoptosis-inducing activity [Thompson, supra].

#### TNF Family of Cytokines

Various molecules, such as tumor necrosis factor- $\alpha$  ("TNF- $\alpha$ "), tumor necrosis factor- $\beta$  ("TNF- $\beta$ " or "lymphotoxin"), CD30 ligand, CD27 ligand, CD40 ligand, OX-40 ligand, 4-1BB ligand, Apo-1 ligand (also referred to as Fas ligand or CD95 ligand), and Apo-2 ligand (also referred to as TRAIL) have been identified as members of the tumor necrosis factor ("TNF") family of cytokines [See, e.g., Gruss and Dower, Blood, 85:3378-3404 (1995); Wiley et al., Immunity, 3:673-682 (1995); Pitti et al., J. Biol. Chem., 271:12687-12690 (1996)]. Among these molecules, TNF- $\alpha$ , TNF- $\beta$ , CD30 ligand, 4-1BB ligand, Apo-1 ligand, and Apo-2 ligand (TRAIL) have been reported to be involved in apoptotic cell death. Both TNF- $\alpha$  and TNF- $\beta$  have been reported to induce apoptotic death in susceptible tumor cells [Schmid et al., Proc. Natl. Acad. Sci., 83:1881 (1986); Dealtry et al., Eur. J. Immunol., 17:689 (1987)]. Zheng et al. have reported that TNF- $\alpha$  is involved in post-stimulation apoptosis of CD8-positive T cells [Zheng et al., Nature, 377:348-351 (1995)]. Other investigators have reported

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that CD30 ligand may be involved in deletion of self-reactive T cells in the thymus [Amakawa et al., Cold Spring Harbor Laboratory Symposium on Programmed Cell Death, Abstr. No. 10, (1995)].

5 Mutations in the mouse Fas/Apo-1 receptor or ligand genes (called *lpr* and *gld*, respectively) have been associated with some autoimmune disorders, indicating that Apo-1 ligand may play a role in regulating the clonal deletion of self-reactive lymphocytes in the periphery [Krammer et al., Curr. Op. Immunol., 6:279-289 (1994); Nagata et al., Science, 267:1449-1456 (1995)]. Apo-1  
10 ligand is also reported to induce post-stimulation apoptosis in CD4-positive T lymphocytes and in B lymphocytes, and may be involved in the elimination of activated lymphocytes when their function is no longer needed [Krammer et al., supra; Nagata et al., supra]. Agonist mouse monoclonal antibodies specifically binding  
15 to the Apo-1 receptor have been reported to exhibit cell killing activity that is comparable to or similar to that of TNF- $\alpha$  [Yonehara et al., J. Exp. Med., 169:1747-1756 (1989)].

#### TNF Family of Receptors

20 Induction of various cellular responses mediated by such TNF family cytokines is believed to be initiated by their binding to specific cell receptors. Two distinct TNF receptors of approximately 55-kDa (TNFR1) and 75-kDa (TNFR2) have been identified [Hohman et al., J. Biol. Chem., 264:14927-14934 (1989); Brockhaus et al., Proc. Natl. Acad. Sci., 87:3127-3131 (1990); EP  
25 417,563, published March 20, 1991] and human and mouse cDNAs corresponding to both receptor types have been isolated and characterized [Loetscher et al., Cell, 61:351 (1990); Schall et al., Cell, 61:361 (1990); Smith et al., Science, 248:1019-1023 (1990); Lewis et al., Proc. Natl. Acad. Sci., 88:2830-2834 (1991);  
30 Goodwin et al., Mol. Cell. Biol., 11:3020-3026 (1991)]. Extensive polymorphisms have been associated with both TNF receptor genes [see, e.g., Takao et al., Immunogenetics, 37:199-203 (1993)]. Both TNFRs share the typical structure of cell surface receptors including extracellular, transmembrane and intracellular regions.  
35 The extracellular portions of both receptors are found naturally

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also as soluble TNF-binding proteins [Nophar, Y. et al., EMBO J., 9:3269 (1990); and Kohno, T. et al., Proc. Natl. Acad. Sci. U.S.A., 87:8331 (1990)]. More recently, the cloning of recombinant soluble TNF receptors was reported by Hale et al. [J. Cell. Biochem. Supplement 15F, 1991, p. 113 (P424)].

The extracellular portion of type 1 and type 2 TNFRs (TNFR1 and TNFR2) contains a repetitive amino acid sequence pattern of four cysteine-rich domains (CRDs) designated 1 through 4, starting from the NH<sub>2</sub>-terminus. Each CRD is about 40 amino acids long and contains 4 to 6 cysteine residues at positions which are well conserved [Schall et al., supra; Loetscher et al., supra; Smith et al., supra; Nophar et al., supra; Kohno et al., supra]. In TNFR1, the approximate boundaries of the four CRDs are as follows: CRD1- amino acids 14 to about 53; CRD2- amino acids from about 54 to about 97; CRD3- amino acids from about 98 to about 138; CRD4- amino acids from about 139 to about 167. In TNFR2, CRD1 includes amino acids 17 to about 54; CRD2- amino acids from about 55 to about 97; CRD3- amino acids from about 98 to about 140; and CRD4- amino acids from about 141 to about 179 [Banner et al., Cell, 73:431-435 (1993)]. The potential role of the CRDs in ligand binding is also described by Banner et al., supra.

A similar repetitive pattern of CRDs exists in several other cell-surface proteins, including the p75 nerve growth factor receptor (NGFR) [Johnson et al., Cell, 47:545 (1986); Radeke et al., Nature, 325:593 (1987)], the B cell antigen CD40 [Stamenkovic et al., EMBO J., 8:1403 (1989)], the T cell antigen OX40 [Mallet et al., EMBO J., 9:1063 (1990)] and the Fas antigen [Yonehara et al., supra and Itoh et al., supra]. CRDs are also found in the soluble TNFR (sTNFR)-like T2 proteins of the Shope and myxoma poxviruses [Upton et al., Virology, 160:20-29 (1987); Smith et al., Biochem. Biophys. Res. Commun., 176:335 (1991); Upton et al., Virology, 184:370 (1991)]. Optimal alignment of these sequences indicates that the positions of the cysteine residues are well conserved. These receptors are sometimes collectively referred to as members of the TNF/NGF receptor superfamily. Recent studies on p75NGFR

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showed that the deletion of CRD1 [Welcher, A.A. et al., Proc. Natl. Acad. Sci. USA, 88:159-163 (1991)] or a 5-amino acid insertion in this domain [Yan, H. and Chao, M.V., J. Biol. Chem., 266:12099-12104 (1991)] had little or no effect on NGF binding [Yan, H. and Chao, M.V., supra]. p75 NGFR contains a proline-rich stretch of about 60 amino acids, between its CRD4 and transmembrane region, which is not involved in NGF binding [Peetre, C. et al., Eur. J. Hematol., 41:414-419 (1988); Seckinger, P. et al., J. Biol. Chem., 264:11966-11973 (1989); Yan, H. and Chao, M.V., supra]. A similar proline-rich region is found in TNFR2 but not in TNFR1.

Itoh et al. disclose that the Apo-1 receptor can signal an apoptotic cell death similar to that signaled by the 55-kDa TNFR1 [Itoh et al., supra]. Expression of the Apo-1 antigen has also been reported to be down-regulated along with that of TNFR1 when cells are treated with either TNF- $\alpha$  or anti-Apo-1 mouse monoclonal antibody [Krammer et al., supra; Nagata et al., supra]. Accordingly, some investigators have hypothesized that cell lines that co-express both Apo-1 and TNFR1 receptors may mediate cell killing through common signaling pathways [Id.].

The TNF family ligands identified to date, with the exception of lymphotoxin- $\alpha$ , are type II transmembrane proteins, whose C-terminus is extracellular. In contrast, the receptors in the TNF receptor (TNFR) family identified to date are type I transmembrane proteins. In both the TNF ligand and receptor families, however, homology identified between family members has been found mainly in the extracellular domain ("ECD"). Several of the TNF family cytokines, including TNF- $\alpha$ , Apo-1 ligand and CD40 ligand, are cleaved proteolytically at the cell surface; the resulting protein in each case typically forms a homotrimeric molecule that functions as a soluble cytokine. TNF receptor family proteins are also usually cleaved proteolytically to release soluble receptor ECDs that can function as inhibitors of the cognate cytokines.

Recently, other members of the TNFR family have been identified. In Marsters et al., Curr. Biol., 6:750 (1996),

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investigators describe a full length native sequence human polypeptide, called Apo-3, which exhibits similarity to the TNFR family in its extracellular cysteine-rich repeats and resembles TNFR1 and CD95 in that it contains a cytoplasmic death domain sequence [see also Marsters et al., Curr. Biol., 6:1669 (1996)]. Apo-3 has also been referred to by other investigators as DR3, wsl-1 and TRAMP [Chinnaiyan et al., Science, 274:990 (1996); Kitson et al., Nature, 384:372 (1996); Bodmer et al., Immunity, 6:79 (1997)].

Pan et al. have disclosed another TNF receptor family member referred to as "DR4" [Pan et al., Science, 276:111-113 (1997)]. The DR4 was reported to contain a cytoplasmic death domain capable of engaging the cell suicide apparatus. Pan et al. disclose that DR4 is believed to be a receptor for the ligand known as Apo-2 ligand or TRAIL.

#### The Apoptosis-Inducing Signaling Complex

As presently understood, the cell death program contains at least three important elements - activators, inhibitors, and effectors; in *C. elegans*, these elements are encoded respectively by three genes, *Ced-4*, *Ced-9* and *Ced-3* [Steller, Science, 267:1445 (1995); Chinnaiyan et al., Science, 275:1122-1126 (1997)]. Two of the TNFR family members, TNFR1 and Fas/Apo1 (CD95), can activate apoptotic cell death [Chinnaiyan and Dixit, Current Biology, 6:555-562 (1996); Fraser and Evan, Cell, 85:781-784 (1996)]. TNFR1 is also known to mediate activation of the transcription factor, NF- $\kappa$ B [Tartaglia et al., Cell, 74:845-853 (1993); Hsu et al., Cell, 84:299-308 (1996)]. In addition to some ECD homology, these two receptors share homology in their intracellular domain (ICD) in an oligomerization interface known as the death domain [Tartaglia et al., supra; Nagata, Cell, 88:355 (1997)]. Death domains are also found in several metazoan proteins that regulate apoptosis, namely, the *Drosophila* protein, Reaper, and the mammalian proteins referred to as FADD/MORT1, TRADD, and RIP [Cleaveland and Ihle, Cell, 81:479-482 (1995)]. Using the yeast-two hybrid system, Raven et al. report the identification of protein, wsl-1, which binds to the TNFR1 death domain [Raven et al., Programmed Cell Death Meeting,

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September 20-24, 1995, Abstract at page 127; Raven et al., European Cytokine Network, 7:Abstr. 82 at page 210 (April-June 1996)]. The wsl-1 protein is described as being homologous to TNFR1 (48% identity) and having a restricted tissue distribution. According to Raven et al., the tissue distribution of wsl-1 is significantly different from the TNFR1 binding protein, TRADD.

Upon ligand binding and receptor clustering, TNFR1 and CD95 are believed to recruit FADD into a death-inducing signalling complex. CD95 purportedly binds FADD directly, while TNFR1 binds FADD indirectly via TRADD [Chinnaiyan et al., Cell, 81:505-512 (1995); Boldin et al., J. Biol. Chem., 270:387-391 (1995); Hsu et al., supra; Chinnaiyan et al., J. Biol. Chem., 271:4961-4965 (1996)]. It has been reported that FADD serves as an adaptor protein which recruits the Ced-3-related protease, MACH $\alpha$ /FLICE (caspase 8), into the death signalling complex [Boldin et al., Cell, 85:803-815 (1996); Muzio et al., Cell, 85:817-827 (1996)]. MACH $\alpha$ /FLICE appears to be the trigger that sets off a cascade of apoptotic proteases, including the interleukin-1 $\beta$  converting enzyme (ICE) and CPP32/Yama, which may execute some critical aspects of the cell death programme [Fraser and Evan, supra].

It was recently disclosed that programmed cell death involves the activity of members of a family of cysteine proteases related to the *C. elegans* cell death gene, *ced-3*, and to the mammalian IL-1-converting enzyme, ICE. The activity of the ICE and CPP32/Yama proteases can be inhibited by the product of the cowpox virus gene, *crmA* [Ray et al., Cell, 69:597-604 (1992); Tewari et al., Cell, 81:801-809 (1995)]. Recent studies show that CrmA can inhibit TNFR1- and CD95-induced cell death [Enari et al., Nature, 375:78-81 (1995); Tewari et al., J. Biol. Chem., 270:3255-3260 (1995)].

As reviewed recently by Tewari et al., TNFR1, TNFR2 and CD40 modulate the expression of proinflammatory and costimulatory cytokines, cytokine receptors, and cell adhesion molecules through activation of the transcription factor, NF- $\kappa$ B [Tewari et al., Curr. Op. Genet. Develop., 6:39-44 (1996)]. NF- $\kappa$ B is the prototype of a

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family of dimeric transcription factors whose subunits contain conserved Rel regions [Verma et al., Genes Develop., 9:2723-2735 (1996); Baldwin, Ann. Rev. Immunol., 14:649-681 (1996)]. In its latent form, NF- $\kappa$ B is complexed with members of the I $\kappa$ B inhibitor family; upon inactivation of the I $\kappa$ B in response to certain stimuli, released NF- $\kappa$ B translocates to the nucleus where it binds to specific DNA sequences and activates gene transcription.

For a review of the TNF family of cytokines and their receptors, see Gruss and Dower, supra.

#### SUMMARY OF THE INVENTION

Applicants have identified cDNA clones that encode novel polypeptides, designated in the present application as "Apo-2." It is believed that Apo-2 is a member of the TNFR family; full-length native sequence human Apo-2 polypeptide exhibits some similarities to some known TNFRs, including a cytoplasmic death domain region. Full-length native sequence human Apo-2 also exhibits similarity to the TNFR family in its extracellular cysteine-rich repeats. Apo-2 polypeptide has been found to be capable of triggering caspase-dependent apoptosis and activating NF- $\kappa$ B. Applicants surprisingly found that the soluble extracellular domain of Apo-2 binds Apo-2 ligand (Apo-2L) and can inhibit Apo-2 ligand function. It is presently believed that Apo-2 ligand can signal via at least two different receptors, DR4 and the newly described Apo-2 herein.

In one embodiment, the invention provides isolated Apo-2 polypeptide. In particular, the invention provides isolated native sequence Apo-2 polypeptide, which in one embodiment, includes an amino acid sequence comprising residues 1 to 411 of Figure 1 (SEQ ID NO:1). In other embodiments, the isolated Apo-2 polypeptide comprises at least about 80% amino acid sequence identity with native sequence Apo-2 polypeptide comprising residues 1 to 411 of Figure 1 (SEQ ID NO:1).

In another embodiment, the invention provides an isolated extracellular domain (ECD) sequence of Apo-2. Optionally, the isolated extracellular domain sequence comprises amino acid

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residues 54 to 182 of Fig. 1 (SEQ ID NO:1).

In another embodiment, the invention provides an isolated death domain sequence of Apo-2. Optionally, the isolated death domain sequence comprises amino acid residues 324 to 391 of Fig. 1 (SEQ ID NO:1).

In another embodiment, the invention provides chimeric molecules comprising Apo-2 polypeptide fused to a heterologous polypeptide or amino acid sequence. An example of such a chimeric molecule comprises an Apo-2 fused to an immunoglobulin sequence. Another example comprises an extracellular domain sequence of Apo-2 fused to a heterologous polypeptide or amino acid sequence, such as an immunoglobulin sequence.

In another embodiment, the invention provides an isolated nucleic acid molecule encoding Apo-2 polypeptide. In one aspect, the nucleic acid molecule is RNA or DNA that encodes an Apo-2 polypeptide or a particular domain of Apo-2, or is complementary to such encoding nucleic acid sequence, and remains stably bound to it under at least moderate, and optionally, under high stringency conditions. In one embodiment, the nucleic acid sequence is selected from:

(a) the coding region of the nucleic acid sequence of Figure 1 (SEQ ID NO:2) that codes for residue 1 to residue 411 (i.e., nucleotides 140-142 through 1370-1372), inclusive;

(b) the coding region of the nucleic acid sequence of Figure 1 (SEQ ID NO:2) that codes for residue 1 to residue 182 (i.e., nucleotides 140-142 through 683-685), inclusive;

(c) the coding region of the nucleic acid sequence of Figure 1 (SEQ ID NO:2) that codes for residue 54 to residue 182 (i.e., nucleotides 299-301 through 6<sup>8</sup>/<sub>3</sub>-6<sup>8</sup>/<sub>5</sub>), inclusive;

(d) the coding region of the nucleic acid sequence of Figure 1 (SEQ ID NO:2) that codes for residue 324 to residue 391 (i.e., nucleotides 1109-1111 through 1310-1312), inclusive; or

(e) a sequence corresponding to the sequence of (a), (b), (c) or (d) within the scope of degeneracy of the genetic code.

In a further embodiment, the invention provides a vector

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comprising the nucleic acid molecule encoding the Apo-2 polypeptide or particular domain of Apo-2. A host cell comprising the vector or the nucleic acid molecule is also provided. A method of producing Apo-2 is further provided.

5 In another embodiment, the invention provides an antibody which specifically binds to Apo-2. The antibody may be an agonistic, antagonistic or neutralizing antibody.

In another embodiment, the invention provides non-human, transgenic or knock-out animals.

10 A further embodiment of the invention provides articles of manufacture and kits that include Apo-2 or Apo-2 antibodies.

#### BRIEF DESCRIPTION OF THE DRAWINGS

15 Figure 1 shows the nucleotide sequence of a native sequence human Apo-2 cDNA and its derived amino acid sequence.

Figure 2A shows the derived amino acid sequence of a native sequence human Apo-2 - the putative signal sequence is underlined, the putative transmembrane domain is boxed, and the putative death domain sequence is dash underlined. The cysteines of the two cysteine-rich domains are individually underlined.

20 Figure 2B shows an alignment and comparison of the death domain sequences of native sequence human Apo-2, DR4, Apo-3/DR3, TNFR1, and Fas/Apo-1 (CD95). Asterisks indicate residues that are essential for death signaling by TNFR1 [Tartaglia et al., supra].

25 Figure 3 shows the interaction of the Apo-2 ECD with Apo-2L. Supernatants from mock-transfected 293 cells or from 293 cells transfected with Flag epitope-tagged Apo-2 ECD were incubated with poly-His-tagged Apo-2L and subjected to immunoprecipitation with anti-Flag conjugated or Nickel conjugated agarose beads. The precipitated proteins were resolved by electrophoresis on polyacrylamide gels, and detected by immunoblot with anti-Apo-2L or anti-Flag antibody.

30 Figure 4 shows the induction of apoptosis by Apo-2 and inhibition of Apo-2L activity by soluble Apo-2 ECD. Human 293 cells (A, B) or HeLa cells (C) were transfected by pRK5 vector or

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by pRK5-based plasmids encoding Apo-2 and/or CrmA. Apoptosis was assessed by morphology (A), DNA fragmentation (B), or by FACS (C-E). Soluble Apo-2L was pre-incubated with buffer or affinity-purified Apo-2 ECD together with anti-Flag antibody or Apo-2 ECD immunoadhesin or DR4 or TNFR1 immunoadhesins and added to HeLa cells. The cells were later analyzed for apoptosis (D). Dose-response analysis using Apo-2L with Apo-2 ECD immunoadhesin was also determined (E).

Figure 5 shows activation of NF- $\kappa$ B by Apo-2, DR4, and Apo-2L. (A) HeLa cells were transfected with expression plasmids encoding the indicated proteins. Nuclear extracts were prepared and analyzed by an electrophoretic mobility shift assay. (B) HeLa cells or MCF7 cells were treated with buffer, Apo-2L or TNF-alpha and assayed for NF- $\kappa$ B activity. (C) HeLa cells were preincubated with buffer, ALLN or cyclohexamide before addition of Apo-2L. Apoptosis was later analyzed by FACS.

Figure 6 shows expression of Apo-2 mRNA in human tissues as analyzed by Northern hybridization of human tissue poly A RNA blots.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

##### I. Definitions

The terms "Apo-2 polypeptide" and "Apo-2" when used herein encompass native sequence Apo-2 and Apo-2 variants (which are further defined herein). These terms encompass Apo-2 from a variety of mammals, including humans. The Apo-2 may be isolated from a variety of sources, such as from human tissue types or from another source, or prepared by recombinant or synthetic methods.

A "native sequence Apo-2" comprises a polypeptide having the same amino acid sequence as an Apo-2 derived from nature. Thus, a native sequence Apo-2 can have the amino acid sequence of naturally-occurring Apo-2 from any mammal. Such native sequence Apo-2 can be isolated from nature or can be produced by recombinant or synthetic means. The term "native sequence Apo-2" specifically encompasses naturally-occurring truncated or secreted forms of the

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Apo-2 (e.g., an extracellular domain sequence), naturally-occurring variant forms (e.g., alternatively spliced forms) and naturally-occurring allelic variants of the Apo-2. A naturally-occurring variant form of the Apo-2 includes an Apo-2 having an amino acid substitution at residue 410 in the amino acid sequence shown in Figure 1 (SEQ ID NO:1). In one embodiment of such naturally-occurring variant form, the leucine residue at position 410 is substituted by a methionine residue. In Fig. 1 (SEQ ID NO:1), the amino acid residue at position 410 is identified as "Xaa" to indicate that the amino acid may, optionally, be either leucine or methionine. In Fig. 1 (SEQ ID NO:2), the nucleotide at position 1367 is identified as "W" to indicate that the nucleotide may be either adenine (A) or thymine (T) or uracil (U). In one embodiment of the invention, the native sequence Apo-2 is a mature or full-length native sequence Apo-2 comprising amino acids 1 to 411 of Fig. 1 (SEQ ID NO:1).

The "Apo-2 extracellular domain" or "Apo-2 ECD" refers to a form of Apo-2 which is essentially free of the transmembrane and cytoplasmic domains of Apo-2. Ordinarily, Apo-2 ECD will have less than 1% of such transmembrane and/or cytoplasmic domains and preferably, will have less than 0.5% of such domains. Optionally, Apo-2 ECD will comprise amino acid residues 54 to 182 of Fig. 1 (SEQ ID NO:1) or amino acid residues 1 to 182 of Fig. 1 (SEQ ID NO:1).

"Apo-2 variant" means a biologically active Apo-2 as defined below having at least about 80% amino acid sequence identity with the Apo-2 having the deduced amino acid sequence shown in Fig. 1 (SEQ ID NO:1) for a full-length native sequence human Apo-2. Such Apo-2 variants include, for instance, Apo-2 polypeptides wherein one or more amino acid residues are added, or deleted, at the N- or C-terminus of the sequence of Fig. 1 (SEQ ID NO:1). Ordinarily, an Apo-2 variant will have at least about 80% amino acid sequence identity, more preferably at least about 90% amino acid sequence identity, and even more preferably at least about 95% amino acid sequence identity with the amino acid sequence

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of Fig. 1 (SEQ ID NO:1).

"Percent (%) amino acid sequence identity" with respect to the Apo-2 sequences identified herein is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the Apo-2 sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as ALIGN™ or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared.

The term "epitope tagged" when used herein refers to a chimeric polypeptide comprising Apo-2, or a domain sequence thereof, fused to a "tag polypeptide". The tag polypeptide has enough residues to provide an epitope against which an antibody can be made, yet is short enough such that it does not interfere with activity of the Apo-2. The tag polypeptide preferably also is fairly unique so that the antibody does not substantially cross-react with other epitopes. Suitable tag polypeptides generally have at least six amino acid residues and usually between about 8 to about 50 amino acid residues (preferably, between about 10 to about 20 residues).

"Isolated," when used to describe the various polypeptides disclosed herein, means polypeptide that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would typically interfere with diagnostic or therapeutic uses for the polypeptide, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In preferred embodiments, the polypeptide will be

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purified (1) to a degree sufficient to obtain at least 15 residues  
of N-terminal or internal amino acid sequence by use of a spinning  
cup sequenator, or (2) to homogeneity by SDS-PAGE under non-  
reducing or reducing conditions using Coomassie blue or,  
5 preferably, silver stain. Isolated polypeptide includes  
polypeptide *in situ* within recombinant cells, since at least one  
component of the Apo-2 natural environment will not be present.  
Ordinarily, however, isolated polypeptide will be prepared by at  
least one purification step.

10 An "isolated" Apo-2 nucleic acid molecule is a nucleic  
acid molecule that is identified and separated from at least one  
contaminant nucleic acid molecule with which it is ordinarily  
associated in the natural source of the Apo-2 nucleic acid. An  
isolated Apo-2 nucleic acid molecule is other than in the form or  
15 setting in which it is found in nature. Isolated Apo-2 nucleic  
acid molecules therefore are distinguished from the Apo-2 nucleic  
acid molecule as it exists in natural cells. However, an isolated  
Apo-2 nucleic acid molecule includes Apo-2 nucleic acid molecules  
contained in cells that ordinarily express Apo-2 where, for  
20 example, the nucleic acid molecule is in a chromosomal location  
different from that of natural cells.

The term "control sequences" refers to DNA sequences  
necessary for the expression of an operably linked coding sequence  
in a particular host organism. The control sequences that are  
25 suitable for prokaryotes, for example, include a promoter,  
optionally an operator sequence, and a ribosome binding site.  
Eukaryotic cells are known to utilize promoters, polyadenylation  
signals, and enhancers.

30 Nucleic acid is "operably linked" when it is placed into  
a functional relationship with another nucleic acid sequence. For  
example, DNA for a presequence or secretory leader is operably  
linked to DNA for a polypeptide if it is expressed as a preprotein  
that participates in the secretion of the polypeptide; a promoter  
or enhancer is operably linked to a coding sequence if it affects  
35 the transcription of the sequence; or a ribosome binding site is

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operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

The term "antibody" is used in the broadest sense and specifically covers single anti-Apo-2 monoclonal antibodies (including agonist, antagonist, and neutralizing antibodies) and anti-Apo-2 antibody compositions with polyepitopic specificity.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally-occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen.

The monoclonal antibodies herein include hybrid and recombinant antibodies produced by splicing a variable (including hypervariable) domain of an anti-Apo-2 antibody with a constant domain (e.g. "humanized" antibodies), or a light chain with a heavy chain, or a chain from one species with a chain from another species, or fusions with heterologous proteins, regardless of species of origin or immunoglobulin class or subclass designation, as well as antibody fragments (e.g., Fab, F(ab')<sub>2</sub>, and Fv), so long as they exhibit the desired biological activity. See, e.g. U.S. Pat. No. 4,816,567 and Mage et al., in Monoclonal Antibody Production Techniques and Applications, pp.79-97 (Marcel Dekker, Inc.: New York, 1987).

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Thus, the modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler and Milstein, Nature, 256:495 (1975), or may be made by recombinant DNA methods such as described in U.S. Pat. No. 4,816,567. The "monoclonal antibodies" may also be isolated from phage libraries generated using the techniques described in McCafferty et al., Nature, 348:552-554 (1990), for example.

"Humanized" forms of non-human (e.g. murine) antibodies are specific chimeric immunoglobulins, immunoglobulin chains, or fragments thereof (such as Fv, Fab, Fab', F(ab')<sub>2</sub> or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat, or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, the humanized antibody may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region or domain (Fc), typically that of a human immunoglobulin.

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"Biologically active" and "desired biological activity" for the purposes herein mean having the ability to modulate apoptosis (either in an agonistic or stimulating manner or in an antagonistic or blocking manner) in at least one type of mammalian cell *in vivo* or *ex vivo*.

The terms "apoptosis" and "apoptotic activity" are used in a broad sense and refer to the orderly or controlled form of cell death in mammals that is typically accompanied by one or more characteristic cell changes, including condensation of cytoplasm, loss of plasma membrane microvilli, segmentation of the nucleus, degradation of chromosomal DNA or loss of mitochondrial function. This activity can be determined and measured, for instance, by cell viability assays, FACS analysis or DNA electrophoresis, all of which are known in the art.

The terms "treating," "treatment," and "therapy" as used herein refer to curative therapy, prophylactic therapy, and preventative therapy.

The term "mammal" as used herein refers to any mammal classified as a mammal, including humans, cows, horses, dogs and cats. In a preferred embodiment of the invention, the mammal is a human.

## II. Compositions and Methods of the Invention

The present invention provides newly identified and isolated Apo-2 polypeptides. In particular, Applicants have identified and isolated various human Apo-2 polypeptides. The properties and characteristics of some of these Apo-2 polypeptides are described in further detail in the Examples below. Based upon the properties and characteristics of the Apo-2 polypeptides disclosed herein, it is Applicants' present belief that Apo-2 is a member of the TNFR family.

A description follows as to how Apo-2, as well as Apo-2 chimeric molecules and anti-Apo-2 antibodies, may be prepared.

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A. Preparation of Apo-2

The description below relates primarily to production of Apo-2 by culturing cells transformed or transfected with a vector containing Apo-2 nucleic acid. It is of course, contemplated that alternative methods, which are well known in the art, may be employed to prepare Apo-2.

1. Isolation of DNA Encoding Apo-2

The DNA encoding Apo-2 may be obtained from any cDNA library prepared from tissue believed to possess the Apo-2 mRNA and to express it at a detectable level. Accordingly, human Apo-2 DNA can be conveniently obtained from a cDNA library prepared from human tissues, such as the bacteriophage libraries of human pancreas and kidney cDNA described in Example 1. The Apo-2-encoding gene may also be obtained from a genomic library or by oligonucleotide synthesis.

Libraries can be screened with probes (such as antibodies to the Apo-2 or oligonucleotides of at least about 20-80 bases) designed to identify the gene of interest or the protein encoded by it. Screening the cDNA or genomic library with the selected probe may be conducted using standard procedures, such as described in Sambrook et al., Molecular Cloning: A Laboratory Manual (New York: Cold Spring Harbor Laboratory Press, 1989). An alternative means to isolate the gene encoding Apo-2 is to use PCR methodology [Sambrook et al., supra; Dieffenbach et al., PCR Primer: A Laboratory Manual (Cold Spring Harbor Laboratory Press, 1995)].

A preferred method of screening employs selected oligonucleotide sequences to screen cDNA libraries from various human tissues. Example 1 below describes techniques for screening a cDNA library. The oligonucleotide sequences selected as probes should be of sufficient length and sufficiently unambiguous that false positives are minimized. The oligonucleotide is preferably labeled such that it can be detected upon hybridization to DNA in the library being screened. Methods of labeling are well known in the art, and include the use of radiolabels like <sup>32</sup>P-labeled ATP, biotinylation or enzyme labeling. Hybridization conditions,

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including moderate stringency and high stringency, are provided in Sambrook et al., supra.

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Nucleic acid having all the protein coding sequence may be obtained by screening selected cDNA or genomic libraries using the deduced amino acid sequence disclosed herein for the first time, and, if necessary, using conventional primer extension procedures as described in Sambrook et al., supra, to detect precursors and processing intermediates of mRNA that may not have been reverse-transcribed into cDNA.

Apo-2 variants can be prepared by introducing appropriate nucleotide changes into the Apo-2 DNA, or by synthesis of the desired Apo-2 polypeptide. Those skilled in the art will appreciate that amino acid changes may alter post-translational processes of the Apo-2, such as changing the number or position of glycosylation sites or altering the membrane anchoring characteristics.

Variations in the native full-length sequence Apo-2 or in various domains of the Apo-2 described herein, can be made, for example, using any of the techniques and guidelines for conservative and non-conservative mutations set forth, for instance, in U.S. Pat. No. 5,364,934. Variations may be a substitution, deletion or insertion of one or more codons encoding the Apo-2 that results in a change in the amino acid sequence of the Apo-2 as compared with the native sequence Apo-2. Optionally the variation is by substitution of at least one amino acid with any other amino acid in one or more of the domains of the Apo-2 molecule. The variations can be made using methods known in the art such as oligonucleotide-mediated (site-directed) mutagenesis, alanine scanning, and PCR mutagenesis. Site-directed mutagenesis [Carter et al., Nucl. Acids Res., 13:4331 (1986); Zoller et al., Nucl. Acids Res., 10:6487 (1987)], cassette mutagenesis [Wells et al., Gene, 34:315 (1985)], restriction selection mutagenesis [Wells et al., Philos. Trans. R. Soc. London SerA, 317:415 (1986)] or other known techniques can be performed on the cloned DNA to produce the Apo-2 variant DNA.

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Scanning amino acid analysis can also be employed to identify one or more amino acids along a contiguous sequence which are involved in the interaction with a particular ligand or receptor. Among the preferred scanning amino acids are relatively small, neutral amino acids. Such amino acids include alanine, glycine, serine, and cysteine. Alanine is the preferred scanning amino acid among this group because it eliminates the side-chain beyond the beta-carbon and is less likely to alter the main-chain conformation of the variant. Alanine is also preferred because it is the most common amino acid. Further, it is frequently found in both buried and exposed positions [Creighton, The Proteins, (W.H. Freeman & Co., N.Y.); Chothia, J. Mol. Biol., 150:1 (1976)]. If alanine substitution does not yield adequate amounts of variant, an isoteric amino acid can be used.

Once selected Apo-2 variants are produced, they can be contacted with, for instance, Apo-2L, and the interaction, if any, can be determined. The interaction between the Apo-2 variant and Apo-2L can be measured by an *in vitro* assay, such as described in the Examples below. While any number of analytical measurements can be used to compare activities and properties between a native sequence Apo-2 and an Apo-2 variant, a convenient one for binding is the dissociation constant  $K_d$  of the complex formed between the Apo-2 variant and Apo-2L as compared to the  $K_d$  for the native sequence Apo-2. Generally, a  $\geq 3$ -fold increase or decrease in  $K_d$  per substituted residue indicates that the substituted residue(s) is active in the interaction of the native sequence Apo-2 with the Apo-2L.

Optionally, representative sites in the Apo-2 sequence suitable for mutagenesis would include sites within the extracellular domain, and particularly, within one or both of the cysteine-rich domains. Such variations can be accomplished using the methods described above.

## 2. Insertion of Nucleic Acid into A Replicable Vector

The nucleic acid (e.g., cDNA or genomic DNA) encoding Apo-2 may be inserted into a replicable vector for further cloning

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(amplification of the DNA) or for expression. Various vectors are publicly available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence, each of which is described below.

(i) Signal Sequence Component

The Apo-2 may be produced recombinantly not only directly, but also as a fusion polypeptide with a heterologous polypeptide, which may be a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. In general, the signal sequence may be a component of the vector, or it may be a part of the Apo-2 DNA that is inserted into the vector. The heterologous signal sequence selected preferably is one that is recognized and processed (i.e., cleaved by a signal peptidase) by the host cell. The signal sequence may be a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion the signal sequence may be, e.g., the yeast invertase leader, alpha factor leader (including *Saccharomyces* and *Kluyveromyces*  $\alpha$ -factor leaders, the latter described in U.S. Pat. No. 5,010,182), or acid phosphatase leader, the *C. albicans* glucoamylase leader (EP 362,179 published 4 April 1990), or the signal described in WO 90/13646 published 15 November 1990. In mammalian cell expression the native Apo-2 presequence that normally directs insertion of Apo-2 in the cell membrane of human cells *in vivo* is satisfactory, although other mammalian signal sequences may be used to direct secretion of the protein, such as signal sequences from secreted polypeptides of the same or related species, as well as viral secretory leaders, for example, the herpes simplex glycoprotein D signal.

The DNA for such precursor region is preferably ligated in reading frame to DNA encoding Apo-2.

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(ii) Origin of Replication Component

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Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2 $\mu$  plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used because it contains the early promoter).

Most expression vectors are "shuttle" vectors, i.e., they are capable of replication in at least one class of organisms but can be transfected into another organism for expression. For example, a vector is cloned in *E. coli* and then the same vector is transfected into yeast or mammalian cells for expression even though it is not capable of replicating independently of the host cell chromosome.

DNA may also be amplified by insertion into the host genome. This is readily accomplished using *Bacillus* species as hosts, for example, by including in the vector a DNA sequence that is complementary to a sequence found in *Bacillus* genomic DNA. Transfection of *Bacillus* with this vector results in homologous recombination with the genome and insertion of Apo-2 DNA. However, the recovery of genomic DNA encoding Apo-2 is more complex than that of an exogenously replicated vector because restriction enzyme digestion is required to excise the Apo-2 DNA.

(iii) Selection Gene Component

Expression and cloning vectors typically contain a selection gene, also termed a selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host

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cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g., the gene encoding D-alanine racemase for *Bacilli*.

One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene produce a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin [Southern et al., J. Molec. Appl. Genet., 1:327 (1982)], mycophenolic acid (Mulligan et al., Science, 209:1422 (1980)] or hygromycin [Sugden et al., Mol. Cell. Biol., 5:410-413 (1985)]. The three examples given above employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug G418 or neomycin (geneticin), xgpt (mycophenolic acid), or hygromycin, respectively.

Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the Apo-2 nucleic acid, such as DHFR or thymidine kinase. The mammalian cell transformants are placed under selection pressure that only the transformants are uniquely adapted to survive by virtue of having taken up the marker. Selection pressure is imposed by culturing the transformants under conditions in which the concentration of selection agent in the medium is successively changed, thereby leading to amplification of both the selection gene and the DNA that encodes Apo-2. Amplification is the process by which genes in greater demand for the production of a protein critical for growth are reiterated in tandem within the chromosomes of successive generations of recombinant cells. Increased quantities of Apo-2 are synthesized from the amplified DNA. Other examples of amplifiable genes include metallothionein-I and -II, adenosine deaminase, and

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ornithine decarboxylase.

Cells transformed with the DHFR selection gene may first be identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub et al., Proc. Natl. Acad. Sci. USA, 77:4216 (1980). The transformed cells are then exposed to increased levels of methotrexate. This leads to the synthesis of multiple copies of the DHFR gene, and, concomitantly, multiple copies of other DNA comprising the expression vectors, such as the DNA encoding Apo-2. This amplification technique can be used with any otherwise suitable host, e.g., ATCC No. CCL61 CHO-K1, notwithstanding the presence of endogenous DHFR if, for example, a mutant DHFR gene that is highly resistant to Mtx is employed (EP 117,060).

Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding Apo-2, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3'-phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, e.g., kanamycin, neomycin, or G418. See U.S. Patent No. 4,965,199.

A suitable selection gene for use in yeast is the *trp1* gene present in the yeast plasmid YRp7 [Stinchcomb et al., Nature, 282:39 (1979); Kingsman et al., Gene, 7:141 (1979); Tschemper et al., Gene, 10:157 (1980)]. The *trp1* gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1 [Jones, Genetics, 85:12 (1977)]. The presence of the *trp1* lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, *Leu2*-deficient yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the *Leu2* gene.

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In addition, vectors derived from the 1.6  $\mu$ m circular plasmid pKD1 can be used for transformation of *Kluyveromyces* yeasts [Bianchi et al., Curr. Genet., 12:185 (1987)]. More recently, an expression system for large-scale production of recombinant calf chymosin was reported for *K. lactis* [Van den Berg, Bio/Technology, 8:135 (1990)]. Stable multi-copy expression vectors for secretion of mature recombinant human serum albumin by industrial strains of *Kluyveromyces* have also been disclosed [Fleer et al., Bio/Technology, 9:968-975 (1991)].

(iv) Promoter Component

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the Apo-2 nucleic acid sequence. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of particular nucleic acid sequence, such as the Apo-2 nucleic acid sequence, to which they are operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, e.g., the presence or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These promoters are operably linked to Apo-2 encoding DNA by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector. Both the native Apo-2 promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of the Apo-2 DNA.

Promoters suitable for use with prokaryotic hosts include the  $\beta$ -lactamase and lactose promoter systems [Chang et al., Nature, 275:615 (1978); Goeddel et al., Nature, 281:544 (1979)], alkaline phosphatase, a tryptophan (trp) promoter system [Goeddel, Nucleic Acids Res., 8:4057 (1980); EP 36,776], and hybrid promoters such as the tac promoter [deBoer et al., Proc. Natl. Acad. Sci. USA, 80:21-

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25 (1983)]. However, other known bacterial promoters are suitable. Their nucleotide sequences have been published, thereby enabling a skilled worker operably to ligate them to DNA encoding Apo-2 [Siebenlist et al., Cell, 20:269 (1980)] using linkers or adaptors to supply any required restriction sites. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding Apo-2.

Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CXCAAT region where X may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into eukaryotic expression vectors.

Examples of suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase [Hitzeman et al., J. Biol. Chem., 255:2073 (1980)] or other glycolytic enzymes [Hess et al., J. Adv. Enzyme Reg., 7:149 (1968); Holland, Biochemistry, 17:4900 (1978)], such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in EP 73,657. Yeast enhancers also are advantageously used with yeast promoters.

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Apo-2 transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published 5 July 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40), from heterologous mammalian promoters, e.g., the actin promoter or an immunoglobulin promoter, from heat-shock promoters, and from the promoter normally associated with the Apo-2 sequence, provided such promoters are compatible with the host cell systems.

The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication [Fiers et al., Nature, 273:113 (1978); Mulligan and Berg, Science, 209:1422-1427 (1980); Pavlakis et al., Proc. Natl. Acad. Sci. USA, 78:7398-7402 (1981)]. The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment [Greenaway et al., Gene, 18:355-360 (1982)]. A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. Patent No. 4,419,446. A modification of this system is described in U.S. Patent No. 4,601,978 [See also Gray et al., Nature, 295:503-508 (1982) on expressing cDNA encoding immune interferon in monkey cells; Reyes et al., Nature, 297:598-601 (1982) on expression of human  $\beta$ -interferon cDNA in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus; Canaani and Berg, Proc. Natl. Acad. Sci. USA 79:5166-5170 (1982) on expression of the human interferon  $\beta$ 1 gene in cultured mouse and rabbit cells; and Gorman et al., Proc. Natl. Acad. Sci. USA, 79:6777-6781 (1982) on expression of bacterial CAT sequences in CV-1 monkey kidney cells, chicken embryo fibroblasts, Chinese hamster ovary cells, HeLa cells, and mouse NIH-3T3 cells using the Rous sarcoma virus long terminal repeat as a promoter].

(v) Enhancer Element Component

Transcription of a DNA encoding the Apo-2 of this

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invention by higher eukaryotes may be increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp, that act on a promoter to increase its transcription. Enhancers are relatively orientation and position independent, having been found 5' [Laimins et al., Proc. Natl. Acad. Sci. USA, 78:993 (1981)] and 3' [Lusky et al., Mol. Cell Bio., 3:1108 (1983)] to the transcription unit, within an intron [Banerji et al., Cell, 33:729 (1983)], as well as within the coding sequence itself [Osborne et al., Mol. Cell Bio., 4:1293 (1984)]. Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin,  $\alpha$ -fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, Nature, 297:17-18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to the Apo-2 coding sequence, but is preferably located at a site 5' from the promoter.

(vi) Transcription Termination Component

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding Apo-2.

(vii) Construction and Analysis of Vectors

Construction of suitable vectors containing one or more of the above-listed components employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and re-ligated in the form desired to generate the

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plasmids required.

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures can be used to transform *E. coli* K12 strain 294 (ATCC 31,446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced by the method of Messing et al., Nucleic Acids Res., 9:309 (1981) or by the method of Maxam et al., Methods in Enzymology, 65:499 (1980).

(viii) Transient Expression Vectors

Expression vectors that provide for the transient expression in mammalian cells of DNA encoding Apo-2 may be employed. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector [Sambrook et al., *supra*]. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of such polypeptides for desired biological or physiological properties. Thus, transient expression systems are particularly useful in the invention for purposes of identifying Apo-2 variants.

(ix) Suitable Exemplary Vertebrate Cell Vectors

Other methods, vectors, and host cells suitable for adaptation to the synthesis of Apo-2 in recombinant vertebrate cell culture are described in Gething et al., Nature, 293:620-625 (1981); Mantei et al., Nature, 281:40-46 (1979); EP 117,060; and EP 117,058.

3. Selection and Transformation of Host Cells

Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include but are not limited to eubacteria, such as Gram-negative or

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Gram-positive organisms, for example, Enterobacteriaceae such as *Escherichia*, e.g., *E. coli*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Proteus*, *Salmonella*, e.g., *Salmonella typhimurium*, *Serratia*, e.g., *Serratia marcescans*, and *Shigella*, as well as *Bacilli* such as *B. subtilis* and *B. licheniformis* (e.g., *B. licheniformis* 41P disclosed in DD 266,710 published 12 April 1989), *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*. Preferably, the host cell should secrete minimal amounts of proteolytic enzymes.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for Apo-2-encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein.

Suitable host cells for the expression of glycosylated Apo-2 are derived from multicellular organisms. Such host cells are capable of complex processing and glycosylation activities. In principle, any higher eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified [See, e.g., Luckow et al., Bio/Technology, 6:47-55 (1988); Miller et al., in Genetic Engineering, Setlow et al., eds., Vol. 8 (Plenum Publishing, 1986), pp. 277-279; and Maeda et al., Nature, 315:592-594 (1985)]. A variety of viral strains for transfection are publicly available, e.g., the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV.

Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can be utilized as hosts. Typically, plant cells are transfected by incubation with certain strains of the bacterium *Agrobacterium tumefaciens*. During incubation of the plant cell culture with *A. tumefaciens*, the DNA encoding the Apo-2

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can be transferred to the plant cell host such that it is transfected, and will, under appropriate conditions, express the Apo-2-encoding DNA. In addition, regulatory and signal sequences compatible with plant cells are available, such as the nopaline synthase promoter and polyadenylation signal sequences [Depicker et al., J. Mol. Appl. Gen., 1:561 (1982)]. In addition, DNA segments isolated from the upstream region of the T-DNA 780 gene are capable of activating or increasing transcription levels of plant-expressible genes in recombinant DNA-containing plant tissue [EP 321,196 published 21 June 1989].

Propagation of vertebrate cells in culture (tissue culture) is also well known in the art [See, e.g., Tissue Culture, Academic Press, Kruse and Patterson, editors (1973)]. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., J. Gen Virol., 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, Proc. Natl. Acad. Sci. USA, 77:4216 (1980)); mouse sertoli cells (TM4, Mather, Biol. Reprod., 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., Annals N.Y. Acad. Sci., 383:44-68 (1982)); MRC 5 cells; and FS4 cells.

Host cells are transfected and preferably transformed with the above-described expression or cloning vectors for Apo-2 production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in

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fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example,  $\text{CaPO}_4$  and electroporation. Successful transfection is generally recognized when any indication of the operation of this vector occurs within the host cell.

5 Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as  
10 described in Sambrook et al., supra, or electroporation is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. Infection with *Agrobacterium tumefaciens* is used for transformation of certain plant cells, as described by Shaw et al., Gene, 23:315 (1983) and WO 89/05859  
15 published 29 June 1989. In addition, plants may be transfected using ultrasound treatment as described in WO 91/00358 published 10 January 1991.

For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb, Virology,  
20 52:456-457 (1978) is preferred. General aspects of mammalian cell host system transformations have been described in U.S. Pat. No. 4,399,216. Transformations into yeast are typically carried out according to the method of Van Solingen et al., J. Bact., 130:946 (1977) and Hsiao et al., Proc. Natl. Acad. Sci. (USA), 76:3829  
25 (1979). However, other methods for introducing DNA into cells, such as by nuclear microinjection, electroporation, bacterial protoplast fusion with intact cells, or polycations, e.g., polybrene, polyornithine, may also be used. For various techniques for transforming mammalian cells, see Keown et al., Methods in  
30 Enzymology, 185:527-537 (1990) and Mansour et al., Nature, 336:348-352 (1988).

#### 4. Culturing the Host Cells

Prokaryotic cells used to produce Apo-2 may be cultured in suitable media as described generally in Sambrook et al., supra.

35 The mammalian host cells used to produce Apo-2 may be

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cultured in a variety of media. Examples of commercially available media include Ham's F10 (Sigma), Minimal Essential Medium ("MEM", Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ("DMEM", Sigma). Any such media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin<sup>TM</sup> drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

In general, principles, protocols, and practical techniques for maximizing the productivity of mammalian cell cultures can be found in Mammalian Cell Biotechnology: a Practical Approach, M. Butler, ed. (IRL Press, 1991).

The host cells referred to in this disclosure encompass cells in culture as well as cells that are within a host animal.

#### 5. Detecting Gene Amplification/Expression

Gene amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA [Thomas, Proc. Natl. Acad. Sci. USA, 77:5201-5205 (1980)], dot blotting (DNA analysis), or *in situ* hybridization, using an appropriately labeled probe, based on the sequences provided herein. Various labels may be employed, most commonly radioisotopes, and particularly <sup>32</sup>P. However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionucleotides, fluorescers or enzymes. Alternatively,

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antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of cells or tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. With immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and fixation, followed by reaction with labeled antibodies specific for the gene product coupled, where the labels are usually visually detectable, such as enzymatic labels, fluorescent labels, or luminescent labels.

Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared in any mammal. Conveniently, the antibodies may be prepared against a native sequence Apo-2 polypeptide or against a synthetic peptide based on the DNA sequences provided herein or against exogenous sequence fused to Apo-2 DNA and encoding a specific antibody epitope.

#### 6. Purification of Apo-2 Polypeptide

Forms of Apo-2 may be recovered from culture medium or from host cell lysates. If the Apo-2 is membrane-bound, it can be released from the membrane using a suitable detergent solution (e.g. Triton-X 100) or its extracellular domain may be released by enzymatic cleavage.

When Apo-2 is produced in a recombinant cell other than one of human origin, the Apo-2 is free of proteins or polypeptides of human origin. However, it may be desired to purify Apo-2 from recombinant cell proteins or polypeptides to obtain preparations that are substantially homogeneous as to Apo-2. As a first step, the culture medium or lysate may be centrifuged to remove particulate cell debris. Apo-2 thereafter is purified from

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contaminant soluble proteins and polypeptides, with the following procedures being exemplary of suitable purification procedures: fractionation on an ion-exchange column; ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; and protein A Sepharose columns to remove contaminants such as IgG.

Apo-2 variants in which residues have been deleted, inserted, or substituted can be recovered in the same fashion as native sequence Apo-2, taking account of changes in properties occasioned by the variation. For example, preparation of an Apo-2 fusion with another protein or polypeptide, e.g., a bacterial or viral antigen, immunoglobulin sequence, or receptor sequence, may facilitate purification; an immunoaffinity column containing antibody to the sequence can be used to adsorb the fusion polypeptide. Other types of affinity matrices also can be used.

A protease inhibitor such as phenyl methyl sulfonyl fluoride (PMSF) also may be useful to inhibit proteolytic degradation during purification, and antibiotics may be included to prevent the growth of adventitious contaminants. One skilled in the art will appreciate that purification methods suitable for native sequence Apo-2 may require modification to account for changes in the character of Apo-2 or its variants upon expression in recombinant cell culture.

#### 7. Covalent Modifications of Apo-2 Polypeptides

Covalent modifications of Apo-2 are included within the scope of this invention. One type of covalent modification of the Apo-2 is introduced into the molecule by reacting targeted amino acid residues of the Apo-2 with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues of the Apo-2.

Derivatization with bifunctional agents is useful for crosslinking Apo-2 to a water-insoluble support matrix or surface for use in the method for purifying anti-Apo-2 antibodies, and

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vice-versa. Derivatization with one or more bifunctional agents will also be useful for crosslinking Apo-2 molecules to generate Apo-2 dimers. Such dimers may increase binding avidity and extend half-life of the molecule *in vivo*. Commonly used crosslinking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidyl-propionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)-dithio]propioimide yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Patent Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

Other modifications include deamidation of glutamyl and asparaginyl residues to the corresponding glutamyl and aspartyl residues, respectively, hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the  $\alpha$ -amino groups of lysine, arginine, and histidine side chains [T.E. Creighton, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)], acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group. The modified forms of the residues fall within the scope of the present invention.

Another type of covalent modification of the Apo-2 polypeptide included within the scope of this invention comprises altering the native glycosylation pattern of the polypeptide. "Altering the native glycosylation pattern" is intended for purposes herein to mean deleting one or more carbohydrate moieties found in native sequence Apo-2, and/or adding one or more glycosylation sites that are not present in the native sequence Apo-2.

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Glycosylation of polypeptides is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-aceylgalactosamine, galactose, or xylose to a hydroxylamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Addition of glycosylation sites to the Apo-2 polypeptide may be accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the native sequence Apo-2 (for O-linked glycosylation sites). The Apo-2 amino acid sequence may optionally be altered through changes at the DNA level, particularly by mutating the DNA encoding the Apo-2 polypeptide at preselected bases such that codons are generated that will translate into the desired amino acids. The DNA mutation(s) may be made using methods described above and in U.S. Pat. No. 5,364,934, supra.

Another means of increasing the number of carbohydrate moieties on the Apo-2 polypeptide is by chemical or enzymatic coupling of glycosides to the polypeptide. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. These methods are described in WO 87/05330 published 11 September 1987, and in Aplin and Wriston, CRC Crit. Rev. Biochem., pp. 259-306 (1981).

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Removal of carbohydrate moieties present on the Apo-2 polypeptide may be accomplished chemically or enzymatically or by mutational substitution of codons encoding for amino acid residues that serve as targets for glycosylation. For instance, chemical deglycosylation by exposing the polypeptide to the compound trifluoromethanesulfonic acid, or an equivalent compound can result in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the polypeptide intact. Chemical deglycosylation is described by Hakimuddin, et al., Arch. Biochem. Biophys., 259:52 (1987) and by Edge et al., Anal. Biochem., 118:131 (1981). Enzymatic cleavage of carbohydrate moieties on polypeptides can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura et al., Meth. Enzymol., 138:350 (1987).

Glycosylation at potential glycosylation sites may be prevented by the use of the compound tunicamycin as described by Duskin et al., J. Biol. Chem., 257:3105 (1982). Tunicamycin blocks the formation of protein-N-glycoside linkages.

Another type of covalent modification of Apo-2 comprises linking the Apo-2 polypeptide to one of a variety of nonproteinaceous polymers, e.g., polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

#### 8. Apo-2 Chimeras

The present invention also provides chimeric molecules comprising Apo-2 fused to another, heterologous polypeptide or amino acid sequence.

In one embodiment, the chimeric molecule comprises a fusion of the Apo-2 with a tag polypeptide which provides an epitope to which an anti-tag antibody can selectively bind. The epitope tag is generally placed at the amino- or carboxyl- terminus of the Apo-2. The presence of such epitope-tagged forms of the Apo-2 can be detected using an antibody against the tag polypeptide. Also, provision of the epitope tag enables the Apo-2

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to be readily purified by affinity purification using an anti-tag antibody or another type of affinity matrix that binds to the epitope tag.

Various tag polypeptides and their respective antibodies are well known in the art. Examples include the flu HA tag polypeptide and its antibody 12CA5 [Field et al., Mol. Cell. Biol., 8:2159-2165 (1988)]; the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto [Evan et al., Molecular and Cellular Biology, 5:3610-3616 (1985)]; and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody [Paborsky et al., Protein Engineering, 3(6):547-553 (1990)]. Other tag polypeptides include the Flag-peptide [Hopp et al., BioTechnology, 6:1204-1210 (1988)]; the KT3 epitope peptide [Martin et al., Science, 255:192-194 (1992)]; an  $\alpha$ -tubulin epitope peptide [Skinner et al., J. Biol. Chem., 266:15163-15166 (1991)]; and the T7 gene 10 protein peptide tag [Lutz-Freyermuth et al., Proc. Natl. Acad. Sci. USA, 87:6393-6397 (1990)]. Once the tag polypeptide has been selected, an antibody thereto can be generated using the techniques disclosed herein.

Generally, epitope-tagged Apo-2 may be constructed and produced according to the methods described above. Epitope-tagged Apo-2 is also described in the Examples below. Apo-2-tag polypeptide fusions are preferably constructed by fusing the cDNA sequence encoding the Apo-2 portion in-frame to the tag polypeptide DNA sequence and expressing the resultant DNA fusion construct in appropriate host cells. Ordinarily, when preparing the Apo-2-tag polypeptide chimeras of the present invention, nucleic acid encoding the Apo-2 will be fused at its 3' end to nucleic acid encoding the N-terminus of the tag polypeptide, however 5' fusions are also possible. For example, a polyhistidine sequence of about 5 to about 10 histidine residues may be fused at the N-terminus or the C-terminus and used as a purification handle in affinity chromatography.

Epitope-tagged Apo-2 can be purified by affinity chromatography using the anti-tag antibody. The matrix to which

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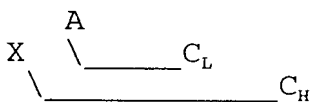
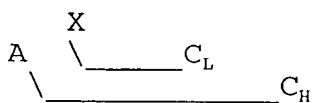
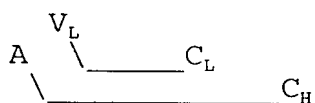
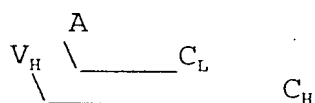
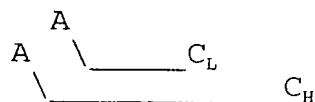
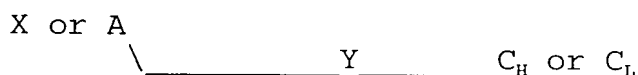
the affinity antibody is attached may include, for instance, agarose, controlled pore glass or poly(styrene-divinyl)benzene. The epitope-tagged Apo-2 can then be eluted from the affinity column using techniques known in the art.

5           In another embodiment, the chimeric molecule comprises an Apo-2 polypeptide fused to an immunoglobulin sequence. The chimeric molecule may also comprise a particular domain sequence of Apo-2, such as the extracellular domain sequence of native Apo-2 fused to an immunoglobulin sequence. This includes chimeras in  
10 monomeric, homo- or heteromultimeric, and particularly homo- or heterodimeric, or -tetrameric forms; optionally, the chimeras may be in dimeric forms or homodimeric heavy chain forms. Generally, these assembled immunoglobulins will have known unit structures as represented by the following diagrams.

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A basic four chain structural unit is the form in which IgG, IgD, and IgE exist. A four chain unit is repeated in the higher molecular weight immunoglobulins; IgM generally exists as a pentamer of basic four-chain units held together by disulfide bonds. IgA globulin, and occasionally IgG globulin, may also exist in a multimeric form in serum. In the case of multimers, each four chain unit may be the same or different.

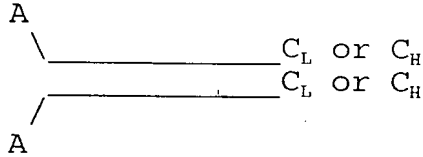
The following diagrams depict some exemplary monomer, homo- and heterodimer and homo- and heteromultimer structures. These diagrams are merely illustrative, and the chains of the

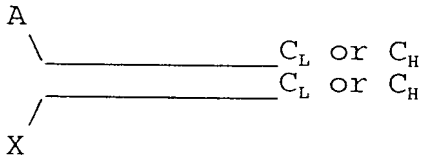
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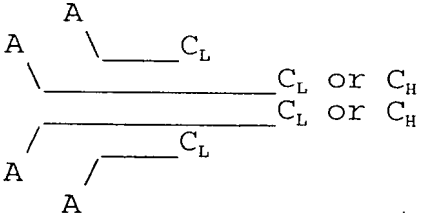
multimers are believed to be disulfide bonded in the same fashion as native immunoglobulins.

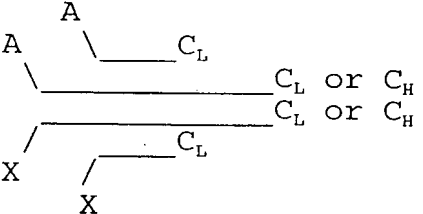
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monomer: A \_\_\_\_\_ C<sub>L</sub> or C<sub>H</sub>

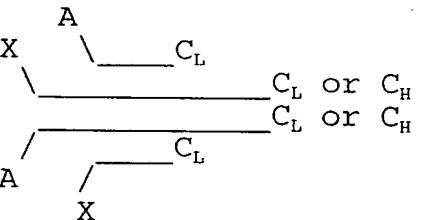
homodimer: 

heterodimer: 

homotetramer: 

heterotetramer: 

and



In the foregoing diagrams, "A" means an Apo-2 sequence or an Apo-2 sequence fused to a heterologous sequence; X is an additional agent, which may be the same as A or different, a

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portion of an immunoglobulin superfamily member such as a variable region or a variable region-like domain, including a native or chimeric immunoglobulin variable region, a toxin such as a pseudomonas exotoxin or ricin, or a sequence functionally binding to another protein, such as other cytokines (i.e., IL-1, interferon- $\gamma$ ) or cell surface molecules (i.e., NGFR, CD40, OX40, Fas antigen, T2 proteins of Shope and myxoma poxviruses), or a polypeptide therapeutic agent not otherwise normally associated with a constant domain; Y is a linker or another receptor sequence; and  $V_L$ ,  $V_H$ ,  $C_L$  and  $C_H$  represent light or heavy chain variable or constant domains of an immunoglobulin. Structures comprising at least one CRD of an Apo-2 sequence as "A" and another cell-surface protein having a repetitive pattern of CRDs (such as TNFR) as "X" are specifically included.

It will be understood that the above diagrams are merely exemplary of the possible structures of the chimeras of the present invention, and do not encompass all possibilities. For example, there might desirably be several different "A"s, "X"s, or "Y"s in any of these constructs. Also, the heavy or light chain constant domains may be originated from the same or different immunoglobulins. All possible permutations of the illustrated and similar structures are all within the scope of the invention herein.

In general, the chimeric molecules can be constructed in a fashion similar to chimeric antibodies in which a variable domain from an antibody of one species is substituted for the variable domain of another species. See, for example, EP 0 125 023; EP 173,494; Munro, Nature, 312:597 (13 December 1984); Neuberger et al., Nature, 312:604-608 (13 December 1984); Sharon et al., Nature, 309:364-367 (24 May 1984); Morrison et al., Proc. Nat'l. Acad. Sci. USA, 81:6851-6855 (1984); Morrison et al., Science, 229:1202-1207 (1985); Boulianne et al., Nature, 312:643-646 (13 December 1984); Capon et al., Nature, 337:525-531 (1989); Traunecker et al., Nature, 339:68-70 (1989).

Alternatively, the chimeric molecules may be constructed

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as follows. The DNA including a region encoding the desired sequence, such as an Apo-2 and/or TNFR sequence, is cleaved by a restriction enzyme at or proximal to the 3' end of the DNA encoding the immunoglobulin-like domain(s) and at a point at or near the DNA encoding the N-terminal end of the Apo-2 or TNFR polypeptide (where use of a different leader is contemplated) or at or proximal to the N-terminal coding region for TNFR (where the native signal is employed). This DNA fragment then is readily inserted proximal to DNA encoding an immunoglobulin light or heavy chain constant region and, if necessary, the resulting construct tailored by deletional mutagenesis. Preferably, the Ig is a human immunoglobulin when the chimeric molecule is intended for *in vivo* therapy for humans. DNA encoding immunoglobulin light or heavy chain constant regions is known or readily available from cDNA libraries or is synthesized. See for example, Adams et al., Biochemistry, 19:2711-2719 (1980); Gough et al., Biochemistry, 19:2702-2710 (1980); Dolby et al., Proc. Natl. Acad. Sci. USA, 77:6027-6031 (1980); Rice et al., Proc. Natl. Acad. Sci., 79:7862-7865 (1982); Falkner et al., Nature, 298:286-288 (1982); and Morrison et al., Ann. Rev. Immunol., 2:239-256 (1984).

Further details of how to prepare such fusions are found in publications concerning the preparation of immunoadhesins. Immunoadhesins in general, and CD4-Ig fusion molecules specifically are disclosed in WO 89/02922, published 6 April 1989). Molecules comprising the extracellular portion of CD4, the receptor for human immunodeficiency virus (HIV), linked to IgG heavy chain constant region are known in the art and have been found to have a markedly longer half-life and lower clearance than the soluble extracellular portion of CD4 [Capon et al., supra; Byrn et al., Nature, 344:667 (1990)]. The construction of specific chimeric TNFR-IgG molecules is also described in Ashkenazi et al. Proc. Natl. Acad. Sci., 88:10535-10539 (1991); Lesslauer et al. [J. Cell. Biochem. Supplement 15F, 1991, p. 115 (P 432)]; and Peppel and Beutler, J. Cell. Biochem. Supplement 15F, 1991, p. 118 (P 439).

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B. Therapeutic and Non-therapeutic Uses for Apo-2

Apo-2, as disclosed in the present specification, can be employed therapeutically to induce apoptosis in mammalian cells. This therapy can be accomplished for instance, using *in vivo* or *ex vivo* gene therapy techniques and includes the use of the death domain sequences disclosed herein. The Apo-2 chimeric molecules (including the chimeric molecules containing the extracellular domain sequence of Apo-2) comprising immunoglobulin sequences can also be employed therapeutically to inhibit apoptosis or NF- $\kappa$ B induction by Apo-2L or by another ligand that Apo-2 binds to.

The Apo-2 of the invention also has utility in non-therapeutic applications. Nucleic acid sequences encoding the Apo-2 may be used as a diagnostic for tissue-specific typing. For example, procedures like *in situ* hybridization, Northern and Southern blotting, and PCR analysis may be used to determine whether DNA and/or RNA encoding Apo-2 is present in the cell type(s) being evaluated. Apo-2 nucleic acid will also be useful for the preparation of Apo-2 by the recombinant techniques described herein.

The isolated Apo-2 may be used in quantitative diagnostic assays as a control against which samples containing unknown quantities of Apo-2 may be prepared. Apo-2 preparations are also useful in generating antibodies, as standards in assays for Apo-2 (e.g., by labeling Apo-2 for use as a standard in a radioimmunoassay, radioreceptor assay, or enzyme-linked immunoassay), in affinity purification techniques, and in competitive-type receptor binding assays when labeled with, for instance, radioiodine, enzymes, or fluorophores.

Modified forms of the Apo-2, such as the Apo-2-IgG chimeric molecules (immunoadhesins) described above, can be used as immunogens in producing anti-Apo-2 antibodies.

Nucleic acids which encode Apo-2 or its modified forms can also be used to generate either transgenic animals or "knock out" animals which, in turn, are useful in the development and screening of therapeutically useful reagents. A transgenic animal

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(e.g., a mouse or rat) is an animal having cells that contain a transgene, which transgene was introduced into the animal or an ancestor of the animal at a prenatal, e.g., an embryonic stage. A transgene is a DNA which is integrated into the genome of a cell from which a transgenic animal develops. In one embodiment, cDNA encoding Apo-2 or an appropriate sequence thereof (such as Apo-2-IgG) can be used to clone genomic DNA encoding Apo-2 in accordance with established techniques and the genomic sequences used to generate transgenic animals that contain cells which express DNA encoding Apo-2. Methods for generating transgenic animals, particularly animals such as mice or rats, have become conventional in the art and are described, for example, in U.S. Patent Nos. 4,736,866 and 4,870,009. Typically, particular cells would be targeted for Apo-2 transgene incorporation with tissue-specific enhancers. Transgenic animals that include a copy of a transgene encoding Apo-2 introduced into the germ line of the animal at an embryonic stage can be used to examine the effect of increased expression of DNA encoding Apo-2. Such animals can be used as tester animals for reagents thought to confer protection from, for example, pathological conditions associated with excessive apoptosis. In accordance with this facet of the invention, an animal is treated with the reagent and a reduced incidence of the pathological condition, compared to untreated animals bearing the transgene, would indicate a potential therapeutic intervention for the pathological condition. In another embodiment, transgenic animals that carry a soluble form of Apo-2 such as the Apo-2 ECD or an immunoglobulin chimera of such form could be constructed to test the effect of chronic neutralization of Apo-2L, a ligand of Apo-2.

Alternatively, non-human homologues of Apo-2 can be used to construct an Apo-2 "knock out" animal which has a defective or altered gene encoding Apo-2 as a result of homologous recombination between the endogenous gene encoding Apo-2 and altered genomic DNA encoding Apo-2 introduced into an embryonic cell of the animal. For example, cDNA encoding Apo-2 can be used to clone genomic DNA encoding Apo-2 in accordance with established techniques. A

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portion of the genomic DNA encoding Apo-2 can be deleted or replaced with another gene, such as a gene encoding a selectable marker which can be used to monitor integration. Typically, several kilobases of unaltered flanking DNA (both at the 5' and 3' ends) are included in the vector [see e.g., Thomas and Capecchi, Cell, 51:503 (1987) for a description of homologous recombination vectors]. The vector is introduced into an embryonic stem cell line (e.g., by electroporation) and cells in which the introduced DNA has homologously recombined with the endogenous DNA are selected [see e.g., Li et al., Cell, 69:915 (1992)]. The selected cells are then injected into a blastocyst of an animal (e.g., a mouse or rat) to form aggregation chimeras [see e.g., Bradley, in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E. J. Robertson, ed. (IRL, Oxford, 1987), pp. 113-152]. A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term to create a "knock out" animal. Progeny harboring the homologously recombined DNA in their germ cells can be identified by standard techniques and used to breed animals in which all cells of the animal contain the homologously recombined DNA. Knockout animals can be characterized for instance, for their ability to defend against certain pathological conditions and for their development of pathological conditions due to absence of the Apo-2 polypeptide, including for example, development of tumors.

C. Anti-Apo-2 Antibody Preparation

The present invention further provides anti-Apo-2 antibodies. Antibodies against Apo-2 may be prepared as follows. Exemplary antibodies include polyclonal, monoclonal, humanized, bispecific, and heteroconjugate antibodies.

1. Polyclonal Antibodies

The Apo-2 antibodies may comprise polyclonal antibodies. Methods of preparing polyclonal antibodies are known to the skilled artisan. Polyclonal antibodies can be raised in a mammal, for example, by one or more injections of an immunizing agent and, if desired, an adjuvant. Typically, the immunizing agent and/or

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adjuvant will be injected in the mammal by multiple subcutaneous or intraperitoneal injections. The immunizing agent may include the Apo-2 polypeptide or a fusion protein thereof. An example of a suitable immunizing agent is a Apo-2-IgG fusion protein or chimeric molecule (including an Apo-2 ECD-IgG fusion protein). Cells expressing Apo-2 at their surface may also be employed. It may be useful to conjugate the immunizing agent to a protein known to be immunogenic in the mammal being immunized. Examples of such immunogenic proteins which may be employed include but are not limited to keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, and soybean trypsin inhibitor. An aggregating agent such as alum may also be employed to enhance the mammal's immune response. Examples of adjuvants which may be employed include Freund's complete adjuvant and MPL-TDM adjuvant (monophosphoryl Lipid A, synthetic trehalose dicorynomycolate). The immunization protocol may be selected by one skilled in the art without undue experimentation. The mammal can then be bled, and the serum assayed for antibody titer. If desired, the mammal can be boosted until the antibody titer increases or plateaus.

## 2. Monoclonal Antibodies

The Apo-2 antibodies may, alternatively, be monoclonal antibodies. Monoclonal antibodies may be prepared using hybridoma methods, such as those described by Kohler and Milstein, supra. In a hybridoma method, a mouse, hamster, or other appropriate host animal, is typically immunized (such as described above) with an immunizing agent to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the immunizing agent. Alternatively, the lymphocytes may be immunized *in vitro*.

The immunizing agent will typically include the Apo-2 polypeptide or a fusion protein thereof. An example of a suitable immunizing agent is a Apo-2-IgG fusion protein or chimeric molecule. Cells expressing Apo-2 at their surface may also be employed. Generally, either peripheral blood lymphocytes ("PBLs") are used if cells of human origin are desired, or spleen cells or

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lymph node cells are used if non-human mammalian sources are desired. The lymphocytes are then fused with an immortalized cell line using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell [Goding, Monoclonal Antibodies: Principles and Practice, Academic Press, (1986) pp. 59-103]. Immortalized cell lines are usually transformed mammalian cells, particularly myeloma cells of rodent, bovine and human origin. Usually, rat or mouse myeloma cell lines are employed. The hybridoma cells may be cultured in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, immortalized cells. For example, if the parental cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine ("HAT medium"), which substances prevent the growth of HGPRT-deficient cells.

Preferred immortalized cell lines are those that fuse efficiently, support stable high level expression of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. More preferred immortalized cell lines are murine myeloma lines, which can be obtained, for instance, from the Salk Institute Cell Distribution Center, San Diego, California and the American Type Culture Collection, Rockville, Maryland. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies [Kozbor, J. Immunol., 133:3001 (1984); Brodeur et al., Monoclonal Antibody Production Techniques and Applications, Marcel Dekker, Inc., New York, (1987) pp. 51-63].

The culture medium in which the hybridoma cells are cultured can then be assayed for the presence of monoclonal antibodies directed against Apo-2. Preferably, the binding specificity of monoclonal antibodies produced by the hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA). Such techniques and assays are

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known in the art. The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson and Pollard, Anal. Biochem., 107:220 (1980).

After the desired hybridoma cells are identified, the clones may be subcloned by limiting dilution procedures and grown by standard methods [Goding, supra]. Suitable culture media for this purpose include, for example, Dulbecco's Modified Eagle's Medium and RPMI-1640 medium. Alternatively, the hybridoma cells may be grown *in vivo* as ascites in a mammal.

The monoclonal antibodies secreted by the subclones may be isolated or purified from the culture medium or ascites fluid by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

The monoclonal antibodies may also be made by recombinant DNA methods, such as those described in U.S. Patent No. 4,816,567. DNA encoding the monoclonal antibodies of the invention can be readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells of the invention serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. The DNA also may be modified, for example, by substituting the coding sequence for human heavy and light chain constant domains in place of the homologous murine sequences [U.S. Patent No. 4,816,567; Morrison et al., supra] or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. Such a non-immunoglobulin polypeptide can be substituted for the constant domains of an antibody of the invention, or can be substituted for the variable domains of one antigen-combining site of an antibody

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of the invention to create a chimeric bivalent antibody.

The antibodies may be monovalent antibodies. Methods for preparing monovalent antibodies are well known in the art. For example, one method involves recombinant expression of immunoglobulin light chain and modified heavy chain. The heavy chain is truncated generally at any point in the Fc region so as to prevent heavy chain crosslinking. Alternatively, the relevant cysteine residues are substituted with another amino acid residue or are deleted so as to prevent crosslinking.

In vitro methods are also suitable for preparing monovalent antibodies. Digestion of antibodies to produce fragments thereof, particularly, Fab fragments, can be accomplished using routine techniques known in the art. For instance, digestion can be performed using papain. Examples of papain digestion are described in WO 94/29348 published 12/22/94 and U.S. Patent No. 4,342,566. Papain digestion of antibodies typically produces two identical antigen binding fragments, called Fab fragments, each with a single antigen binding site, and a residual Fc fragment. Pepsin treatment yields an  $F(ab')_2$  fragment that has two antigen combining sites and is still capable of cross-linking antigen.

The Fab fragments produced in the antibody digestion also contain the constant domains of the light chain and the first constant domain ( $CH_1$ ) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain  $CH_1$  domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group.  $F(ab')_2$  antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

### 3. Humanized Antibodies

The Apo-2 antibodies of the invention may further comprise humanized antibodies or human antibodies. Humanized forms of non-human (e.g., murine) antibodies are chimeric

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immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab')<sub>2</sub>, or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin [Jones et al., Nature, 321:522-525 (1986); Reichmann et al., Nature, 332:323-329 (1988); and Presta, Curr. Op. Struct. Biol., 2:593-596 (1992)].

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers [Jones et al., Nature, 321:522-525 (1986); Reichmann et al., Nature, 332:323-327 (1988); Verhoeven et al., Science, 239:1534-1536 (1988)], by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Patent No. 4,816,567),

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wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important in order to reduce antigenicity. According to the "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody [Sims et al., J. Immunol., 151:2296 (1993); Chothia and Lesk, J. Mol. Biol., 196:901 (1987)]. Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies [Carter et al., Proc. Natl. Acad. Sci. USA, 89:4285 (1992); Presta et al., J. Immunol., 151:2623 (1993)].

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three dimensional models of the parental and humanized sequences. Three dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can

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be selected and combined from the consensus and import sequence so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding [see, WO 94/04679 published 3 March 1994].

Transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production can be employed. For example, it has been described that the homozygous deletion of the antibody heavy chain joining region ( $J_H$ ) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge [see, e.g., Jakobovits et al., Proc. Natl. Acad. Sci. USA, 90:2551-255 (1993); Jakobovits et al., Nature, 362:255-258 (1993); Bruggermann et al., Year in Immuno., 7:33 (1993)]. Human antibodies can also be produced in phage display libraries [Hoogenboom and Winter, J. Mol. Biol., 227:381 (1991); Marks et al., J. Mol. Biol., 222:581 (1991)]. The techniques of Cote et al. and Boerner et al. are also available for the preparation of human monoclonal antibodies (Cote et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985) and Boerner et al., J. Immunol., 147(1):86-95 (1991)].

#### 4. Bispecific Antibodies

Bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens. In the present case, one of the binding specificities is for the Apo-2, the other one is for any other antigen, and preferably for a cell-surface protein or receptor or receptor subunit.

Methods for making bispecific antibodies are known in the art. Traditionally, the recombinant production of bispecific antibodies is based on the co-expression of two immunoglobulin heavy-chain/light-chain pairs, where the two heavy chains have

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different specificities [Millstein and Cuello, Nature, 305:537-539 (1983)]. Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of ten different antibody molecules, of which only one has the correct bispecific structure. The purification of the correct molecule is usually accomplished by affinity chromatography steps. Similar procedures are disclosed in WO 93/08829, published 13 May 1993, and in Traunecker et al., EMBO J., 10:3655-3659 (1991).

According to a different and more preferred approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy-chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light-chain binding present in at least one of the fusions. DNAs encoding the immunoglobulin heavy-chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance. In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy-chain/light-chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in

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only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690 published 3 March 1994. For further details of generating bispecific antibodies see, for example, Suresh et al., Methods in Enzymology, 121:210 (1986).

#### 5. Heteroconjugate Antibodies

Heteroconjugate antibodies are also within the scope of the present invention. Heteroconjugate antibodies are composed of two covalently joined antibodies. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells [US Patent No. 4,676,980], and for treatment of HIV infection [WO 91/00360; WO 92/200373; EP 03089]. It is contemplated that the antibodies may be prepared *in vitro* using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins may be constructed using a disulfide exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptobutyrimidate and those disclosed, for example, in U.S. Pat. No. 4,676,980.

#### D. Therapeutic and Non-therapeutic Uses for Apo-2 Antibodies

The Apo-2 antibodies of the invention have therapeutic utility. Agonistic Apo-2 antibodies, for instance, may be employed to activate or stimulate apoptosis in cancer cells. Alternatively, antagonistic antibodies may be used to block excessive apoptosis (for instance in neurodegenerative disease) or to block potential autoimmune/inflammatory effects of Apo-2 resulting from NF- $\kappa$ B activation.

Apo-2 antibodies may further be used in diagnostic assays for Apo-2, e.g., detecting its expression in specific cells, tissues, or serum. Various diagnostic assay techniques known in the art may be used, such as competitive binding assays, direct or indirect sandwich assays and immunoprecipitation assays conducted in either heterogeneous or homogeneous phases [Zola, Monoclonal Antibodies: A Manual of Techniques, CRC Press, Inc. (1987) pp. 147-158]. The antibodies used in the diagnostic assays can be labeled

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with a detectable moiety. The detectable moiety should be capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety may be a radioisotope, such as  $^3\text{H}$ ,  $^{14}\text{C}$ ,  $^{32}\text{P}$ ,  $^{35}\text{S}$ , or  $^{125}\text{I}$ , a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin, or an enzyme, such as alkaline phosphatase, beta-galactosidase or horseradish peroxidase. Any method known in the art for conjugating the antibody to the detectable moiety may be employed, including those methods described by Hunter et al., Nature, 144:945 (1962); David et al., Biochemistry, 13:1014 (1974); Pain et al., J. Immunol. Meth., 40:219 (1981); and Nygren, J. Histochem. and Cytochem., 30:407 (1982).

Apo-2 antibodies also are useful for the affinity purification of Apo-2 from recombinant cell culture or natural sources. In this process, the antibodies against Apo-2 are immobilized on a suitable support, such a Sephadex resin or filter paper, using methods well known in the art. The immobilized antibody then is contacted with a sample containing the Apo-2 to be purified, and thereafter the support is washed with a suitable solvent that will remove substantially all the material in the sample except the Apo-2, which is bound to the immobilized antibody. Finally, the support is washed with another suitable solvent that will release the Apo-2 from the antibody.

#### E. Kits Containing Apo-2 or Apo-2 Antibodies

In a further embodiment of the invention, there are provided articles of manufacture and kits containing Apo-2 or Apo-2 antibodies which can be used, for instance, for the therapeutic or non-therapeutic applications described above. The article of manufacture comprises a container with a label. Suitable containers include, for example, bottles, vials, and test tubes. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which includes an active agent that is effective for therapeutic or non-therapeutic applications, such as described above. The active agent in the composition is Apo-2 or an Apo-2 antibody. The label

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on the container indicates that the composition is used for a specific therapy or non-therapeutic application, and may also indicate directions for either *in vivo* or *in vitro* use, such as those described above.

5           The kit of the invention will typically comprise the container described above and one or more other containers comprising materials desirable from a commercial and user standpoint, including buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

10           \*\*\*\*\*

The following examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

15           All patent and literature references cited in the present specification are hereby incorporated by reference in their entirety.

#### EXAMPLES

20           All restriction enzymes referred to in the examples were purchased from New England Biolabs and used according to manufacturer's instructions. All other commercially available reagents referred to in the examples were used according to manufacturer's instructions unless otherwise indicated. The source of those cells identified in the following examples, and throughout the specification, by ATCC accession numbers is the American Type Culture Collection, Rockville, Maryland.

#### EXAMPLE 1

##### Isolation of cDNA clones Encoding Human Apo-2

30           Expressed sequence tag (EST) DNA databases (Incyte Pharmaceuticals, Palo Alto, CA) were searched and an EST was identified which showed homology to the death domain of the Apo-3 receptor [Marsters et al., Curr. Biol., 6:750 (1996)]. Human pancreas and kidney lgt10 bacteriophage cDNA libraries (both purchased from Clontech) were ligated into pRK5 vectors as follows.

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Reagents were added together and incubated at 16°C for 16 hours: 5X  
T4 ligase buffer (3 ml); pRK5, Xho1, Not1 digested vector, 0.5 mg,  
1 ml); cDNA (5 ml) and distilled water (6 ml). Subsequently,  
5 additional distilled water (70 ml) and 10 mg/ml tRNA (0.1 ml) were  
added and the entire reaction was extracted through  
phenol:chloroform:isoamyl alcohol (25:24:1). The aqueous phase was  
removed, collected and diluted into 5M NaCl (10 ml) and absolute  
ethanol (-20°C, 250 ml). This was then centrifuged for 20 minutes  
10 at 14,000 x g, decanted, and the pellet resuspended into 70%  
ethanol (0.5 ml) and centrifuged again for 2 minutes at 14,000 x g.  
The DNA pellet was then dried in a speedvac and eluted into  
distilled water (3 ml) for use in the subsequent procedure.

The ligated cDNA/pRK5 vector DNA prepared previously was  
chilled on ice to which was added electrocompetent DH10B bacteria  
15 (Life Tech., 20 ml). The bacteria vector mixture was then  
electroporated as per the manufacturers recommendation.  
Subsequently SOC media (1 ml) was added and the mixture was  
incubated at 37°C for 30 minutes. The transformants were then  
plated onto 20 standard 150 mm LB plates containing ampicillin and  
incubated for 16 hours (37°C) to allow the colonies to grow.  
20 Positive colonies were then scraped off and the DNA isolated from  
the bacterial pellet using standard CsCl-gradient protocols.

An enriched 5'-cDNA library was then constructed to  
obtain a bias of cDNA fragments which preferentially represents the  
25 5' ends of cDNA's contained within the library. 10 mg of the  
pooled isolated full-length library plasmid DNA (41 ml) was  
combined with Not 1 restriction buffer (New England Biolabs, 5 ml)  
and Not 1 (New England Biolabs, 4 ml) and incubated at 37°C for one  
hour. The reaction was extracted through phenol:chloroform:isoamyl  
30 alcohol (25:24:1, 50 ml), the aqueous phase removed, collected and  
resuspended into 5M NaCl (5 ml) and absolute ethanol (-20°C, 150  
ml). This was then centrifuged for 20 minutes at 14,000 x g,  
decanted, resuspended into 70% ethanol (0.5 ml) and centrifuged  
again for 2 minutes at 14,000 x g. The supernatant was then  
35 removed, the pellet dried in a speedvac and resuspended in

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distilled water (10 ml).

5 The following reagents were brought together and incubated at 37°C for 2 hours: distilled water (3 ml); linearized DNA library (1 mg, 1 ml); Ribonucleotide mix (Invitrogen, 10 ml); transcription buffer (Invitrogen, 2 ml) and Sp6 enzyme mix. The reaction was then extracted through phenol:chloroform:isoamyl alcohol (25:24:1, 50 ml) and the aqueous phase was removed, collected and resuspended into 5M NaCl (5 ml) and absolute ethanol (-20°C, 150 ml) and centrifuged for 20 minutes at 14,000 x g. The pellet was then decanted and resuspended in 70% ethanol (0.5 ml), centrifuged again for 2 minutes at 14,000 x g, decanted, dried in a speedvac and resuspended into distilled water (10 ml).

15 The following reagents were added together and incubated at 16°C for 16 hours: 5X T4 ligase buffer (Life Tech., 3 ml); pRK5 Cla-Sal digested vector, 0.5 mg, 1 ml); cDNA (5 ml); distilled water (6 ml). Subsequently, additional distilled water (70 ml) and 10 mg/ml tRNA (0.1 ml) was added and the entire reaction was extracted through phenol:chloroform:isoamyl alcohol (25:24:1, 100 ml). The aqueous phase was removed, collected and diluted by 5M NaCl (10 ml) and absolute ethanol (-20°C, 250 ml) and centrifuged for 20 minutes at 14,000 x g. The DNA pellet was decanted, resuspended into 70% ethanol (0.5 ml) and centrifuged again for 2 minutes at 14,000 x g. The supernatant was removed and the residue pellet was dried in a speedvac and resuspended in distilled water (3ml). The ligated cDNA/pSST-amy.1 vector DNA was chilled on ice to which was added electrocompetent DH10B bacteria (Life Tech., 20 ml). The bacteria vector mixture was then electroporated as recommended by the manufacturer. Subsequently, SOC media (Life Tech., 1 ml) was added and the mixture was incubated at 37°C for 30 minutes. The transformants were then plated onto 20 standard 150 mm LB plates containing ampicillin and incubated for 16 hours (37°C). Positive colonies were scraped off the plates and the DNA was isolated from the bacterial pellet using standard protocols, e.g. CsCl-gradient.

35 The cDNA libraries were screened by hybridization with a

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synthetic oligonucleotide probe:

GGGAGCCGCTCATGAGGAAGTTGGGCCTCATGGACAATGAGATAAAGGTGGCTAAAGCTGAGGCA  
GCGGG (SEQ ID NO:3) based on the EST.

Three cDNA clones were sequenced in entirety. The overlapping coding regions of the cDNAs were identical except for codon 410 (using the numbering system for Fig. 1); this position encoded a leucine residue (TTG) in both pancreatic cDNAs, and a methionine residue (ATG) in the kidney cDNA, possibly due to polymorphism.

The entire nucleotide sequence of Apo-2 is shown in Figure 1 (SEQ ID NO:2). Clone 27868 (also referred to as pRK5-Apo-2 deposited as ATCC \_\_\_\_\_, as indicated below) contains a single open reading frame with an apparent translational initiation site at nucleotide positions 140-142 and ending at the stop codon found at nucleotide positions 1373-1375 (Fig. 1; SEQ ID NO:2) [Kozak et al., supra].

The predicted polypeptide precursor is 411 amino acids long, a type I transmembrane protein, and has a calculated molecular weight of approximately 45 kDa. Hydropathy analysis (not shown) suggested the presence of a signal sequence (residues 1-53), followed by an extracellular domain (residues 54-182), a transmembrane domain (residues 183-208), and an intracellular domain (residues 209-411) (Fig. 2A; SEQ ID NO:1). N-terminal amino acid sequence analysis of Apo-2-IgG expressed in 293 cells showed that the mature polypeptide starts at amino acid residue 54, indicating that the actual signal sequence comprises residues 1-53.

TNF receptor family proteins are typically characterized by the presence of multiple (usually four) cysteine-rich domains in their extracellular regions -- each cysteine-rich domain being approximately 45 amino acids long and containing approximately 6, regularly spaced, cysteine residues. Based on the crystal structure of the type 1 TNF receptor, the cysteines in each domain typically form three disulfide bonds in which usually cysteines 1 and 2, 3 and 5, and 4 and 6 are paired together. Like DR4, Apo-2 contains two extracellular cysteine-rich pseudorepeats (Fig. 2A), whereas other identified mammalian TNFR family members contain

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three or more such domains [Smith et al., Cell, 76:959 (1994)].

The cytoplasmic region of Apo-2 contains a death domain (amino acid residues 324-391 shown in Fig. 1; see also Fig. 2A) which shows significantly more amino acid sequence identity to the death domain of DR4 (64%) than to the death domain of TNFR1 (30%); CD95 (19%); or Apo-3/DR3 (29%) (Fig. 2B). Four out of six death domain amino acids that are required for signaling by TNFR1 [Tartaglia et al., supra] are conserved in Apo-2 while the other two residues are semi-conserved (see Fig. 2B).

Based on an alignment analysis (using the ALIGN™ computer program) of the full-length sequence, Apo-2 shows more sequence identity to DR4 (55%) than to other apoptosis-linked receptors, such as TNFR1 (19%); CD95 (17%); or Apo-3 (also referred to as DR3, WSL-1 or TRAMP) (29%).

## EXAMPLE 2

### A. Expression of Apo-2 ECD

A soluble extracellular domain (ECD) fusion construct was prepared. An Apo-2 ECD (amino acid residues 1-184 shown in Figure 1) was obtained by PCR and fused to a C-terminal Flag epitope tag (Sigma). (The Apo-2 ECD construct included residues 183 and 184 shown in Figure 1 to provide flexibility at the junction, even though residues 183 and 184 are predicted to be in the transmembrane region.) The Flag epitope-tagged molecule was then inserted into pRK5, and expressed by transient transfection into human 293 cells (ATCC CRL 1573).

After a 48 hour incubation, the cell supernatants were collected and either used directly for co-precipitation studies (see Example 3) or subjected to purification of the Apo-2 ECD-Flag by affinity chromatography on anti-Flag agarose beads, according to manufacturer's instructions (Sigma).

### B. Expression of Apo-2 ECD as an Immunoadhesin

A soluble Apo-2 ECD immunoadhesin construct was prepared. The Apo-2 ECD (amino acids 1-184 shown in Fig. 1) was fused to the

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hinge and Fc region of human immunoglobulin G<sub>1</sub> heavy chain in pRK5 as described previously [Ashkenazi et al., Proc. Natl. Acad. Sci., 88:10535-10539 (1991)]. The immunoadhesin was expressed by transient transfection into human 293 cells and purified from cell supernatants by protein A affinity chromatography, as described by Ashkenazi et al., supra.

### EXAMPLE 3

#### Immunoprecipitation Assay Showing Binding Interaction Between Apo-2 and Apo-2 Ligand

To determine whether Apo-2 and Apo-2L interact or associate with each other, supernatants from mock-transfected 293 cells or from 293 cells transfected with Apo-2 ECD-Flag (described in Example 2 above) (5 ml) were incubated with 5 µg poly-histidine-tagged soluble Apo-2L [Pitti et al., supra] for 30 minutes at room temperature and then analyzed for complex formation by a co-precipitation assay.

The samples were subjected to immunoprecipitation using 25 µl anti-Flag conjugated agarose beads (Sigma) or Nickel-conjugated agarose beads (Qiagen). After a 1.5 hour incubation at 4° C, the beads were spun down and washed four times in phosphate buffered saline (PBS). By using anti-Flag agarose, the Apo-2L was precipitated through the Flag-tagged Apo-2 ECD; by using Nickel-agarose, the Apo-2 ECD was precipitated through the His-tagged Apo-2L. The precipitated proteins were released by boiling the beads for 5 minutes in SDS-PAGE buffer, resolved by electrophoresis on 12% polyacrylamide gels, and then detected by immunoblot with anti-Apo-2L or anti-Flag antibody (2 µg/ml) as described in Marsters et al., J. Biol. Chem., **in press** (1997). 3

The results, shown in Figure 2A, indicate that the Apo-2 ECD and Apo-2L can associate with each other.

The binding interaction was further analyzed by purifying Apo-2 ECD from the transfected 293 cell supernatants with anti-Flag beads (see Example 2) and then analyzing the samples on a BIAcore™ instrument. The BIAcore™ analysis indicated a dissociation



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constant ( $K_d$ ) of about 1 nM. BIACORE™ analysis also showed that the Apo-2 ECD is not capable of binding other apoptosis-inducing TNF family members, namely, TNF-alpha (Genentech, Inc., Pennica et al., Nature, 312:712 (1984), lymphotoxin-alpha (Genentech, Inc.), or Fas/Apo-1 ligand (Alexis Biochemicals). The data thus shows that Apo-2 is a specific receptor for Apo-2L.

#### EXAMPLE 4

##### Induction of Apoptosis by Apo-2

Because death domains can function as oligomerization interfaces, over-expression of receptors that contain death domains may lead to activation of signaling in the absence of ligand [Frazer et al., supra, Nagata et al., supra]. To determine whether Apo-2 was capable of inducing cell death, human 293 cells or HeLa cells (ATCC CCL 2.2) were transiently transfected by calcium phosphate precipitation (293 cells) or electroporation (HeLa cells) with a pRK5 vector or pRK5-based plasmids encoding Apo-2 and/or CrmA. When applicable, the total amount of plasmid DNA was adjusted by adding vector DNA. Apoptosis was assessed 24 hours after transfection by morphology (Fig. 4A); DNA fragmentation (Fig. 4B); or by FACS analysis of phosphatidylserine exposure (Fig. 4C) as described in Marsters et al., Curr. Biol., 6:1669 (1996). As shown in Figs. 4A and 4B, the Apo-2 transfected 293 cells underwent marked apoptosis.

For samples assayed by FACS, the HeLa cells were co-transfected with pRK5-CD4 as a marker for transfection and apoptosis was determined in CD4-expressing cells; FADD was co-transfected with the Apo-2 plasmid; the data are means  $\pm$  SEM of at least three experiments, as described in Marsters et al., Curr. Biol., 6:1669 (1996). The caspase inhibitors, DEVD-fmk (Enzyme Systems) or z-VAD-fmk (Research Biochemicals Intl.) were added at 200  $\mu$ M at the time of transfection. As shown in Fig. 4C, the caspase inhibitors CrmA, DEVD-fmk, and z-VAD-fmk blocked apoptosis induction by Apo-2, indicating the involvement of Ced-3-like proteases in this response.

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FADD is an adaptor protein that mediates apoptosis activation by CD95, TNFR1, and Apo-3/DR3 [Nagata et al., supra], but does not appear necessary for apoptosis induction by Apo-2L [Marsters et al., supra] or by DR4 [Pan et al., supra]. A dominant-negative mutant form of FADD, which blocks apoptosis induction by CD95, TNFR1, or Apo-3/DR3 [Frazer et al., supra; Nagata et al., supra; Chinnayian et al., supra] did not inhibit apoptosis induction by Apo-2 when co-transfected into HeLa cells with Apo-2 (Fig. 4C). These results suggest that Apo-2 signals apoptosis independently of FADD. Consistent with this conclusion, a glutathione-S-transferase fusion protein containing the Apo-2 cytoplasmic region did not bind to *in vitro* transcribed and translated FADD (data not shown).

#### EXAMPLE 5

##### Inhibition of Apo-2L Activity by Soluble Apo-2 ECD

Soluble Apo-2L (0.5  $\mu$ g/ml, prepared as described in Pitti et al., supra) was pre-incubated for 1 hour at room temperature with PBS buffer or affinity-purified Apo-2 ECD (5  $\mu$ g/ml) together with anti-Flag antibody (Sigma) (1  $\mu$ g/ml) and added to HeLa cells. After a 5 hour incubation, the cells were analyzed for apoptosis by FACS (as above) (Fig. 4D).

Apo-2L induced marked apoptosis in HeLa cells, and the soluble Apo-2 ECD was capable of blocking Apo-2L action (Fig. 4D), confirming a specific interaction between Apo-2L and Apo-2. Similar results were obtained with the Apo-2 ECD immunoadhesin (Fig. 4D). Dose-response analysis showed half-maximal inhibition at approximately 0.3 nM Apo-2 immunoadhesin (Fig. 4E).

#### EXAMPLE 6

##### Activation of NF- $\kappa$ B by Apo-2

An assay was conducted to determine whether Apo-2 activates NF- $\kappa$ B.

HeLa cells were transfected with pRK5 expression plasmids encoding full-length native sequence Apo-2, DR4 or Apo-3 and

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harvested 24 hours after transfection. Nuclear extracts were prepared and 1  $\mu$ g of nuclear protein was reacted with a  $^{32}$ P-labelled NF- $\kappa$ B-specific synthetic oligonucleotide probe

ATCAGGGACTTTCGCTGGGGACTTTCG (SEQ ID NO:4) [see, also, MacKay et al., J. Immunol., 153:5274-5284 (1994)], alone or together with a 50-fold excess of unlabelled probe, or with an irrelevant  $^{32}$ P-labelled synthetic oligonucleotide

AGGATGGGAAGTGTGTGATATATCCTTGAT (SEQ ID NO:5). In some samples, antibody to p65/RelA subunits of NF- $\kappa$ B (1  $\mu$ g/ml; Santa Cruz Biotechnology) was added. DNA binding was analyzed by an electrophoretic mobility shift assay as described by Hsu et al., supra; Marsters et al., supra, and MacKay et al., supra.

The results are shown in Fig. 5. As shown in Fig. 5A, upon transfection into HeLa cells, both Apo-2 and DR4 induced significant NF- $\kappa$ B activation as measured by the electrophoretic mobility shift assay; the level of activation was comparable to activation observed for Apo-3/DR3. Antibody to the p65/RelA subunit of NF- $\kappa$ B inhibited the mobility of the NF- $\kappa$ B probe, implicating p65 in the response to all 3 receptors.

An assay was also conducted to determine if Apo-2L itself can regulate NF- $\kappa$ B activity. HeLa cells or MCF7 cells (human breast adenocarcinoma cell line, ATCC HTB 22) were treated with PBS buffer, soluble Apo-2L (Pitti et al., supra) or TNF-alpha (Genentech, Inc., see Pennica et al., Nature, 312:721 (1984)) (1  $\mu$ g/ml) and assayed for NF- $\kappa$ B activity as above. The results are shown in Fig. 5B. The Apo-2L induced a significant NF- $\kappa$ B activation in the treated HeLa cells but not in the treated MCF7 cells; the TNF-alpha induced a more pronounced activation in both cell lines. Several studies have disclosed that NF- $\kappa$ B activation by TNF can protect cells against TNF-induced apoptosis [Nagata, supra].

The effects of a NF- $\kappa$ B inhibitor, ALLN (N-acetyl-Leu-Leu-norleucinal) and a transcription inhibitor, cyclohexamide, were also tested. The HeLa cells (<sup>plated</sup> placed in 6-well dishes) were preincubated with PBS buffer, ALLN (Calbiochem) (40  $\mu$ g/ml) or

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cyclohexamide (Sigma) (50  $\mu$ g/ml) for 1 hour before addition of Apo-2L (1  $\mu$ g/ml). After a 5 hour incubation, apoptosis was analyzed by FACS (see Fig. 5C).

The results are shown in Fig. 5C. Both ALLN and cyclohexamide increased the level of Apo-2L-induced apoptosis in the HeLa cells. The data indicates that Apo-2L can induce protective NF- $\kappa$ B-dependent genes. The data also indicates that Apo-2L is capable of activating NF- $\kappa$ B in certain cell lines and that both Apo-2 and DR4 may mediate that function.

#### EXAMPLE 7

##### Northern Blot Analysis

Expression of Apo-2 mRNA in human tissues was examined by Northern blot analysis. Human RNA blots were hybridized to a 4.6 kilobase  $^{32}$ P-labelled DNA probe based on the full length Apo-2 cDNA; the probe was generated by digesting the pRK5-Apo-2 plasmid with EcoRI. Human fetal RNA blot MTN (Clontech) and human adult RNA blot MTN-II (Clontech) were incubated with the DNA probes. Blots were incubated with the probes in hybridization buffer (5X SSPE; 2X Denhardt's solution; 100 mg/mL denatured sheared salmon sperm DNA; 50% formamide; 2% SDS) for 60 hours at 42°C. The blots were washed several times in 2X SSC; 0.05% SDS for 1 hour at room temperature, followed by a 30 minute wash in 0.1X SSC; 0.1% SDS at 50°C. The blots were developed after overnight exposure.

As shown in Fig. 6, a predominant mRNA transcript of approximately 4.6kb was detected in multiple tissues. Expression was relatively high in fetal and adult liver and lung, and in adult ovary and peripheral blood leukocytes (PBL), while no mRNA expression was detected in fetal and adult brain. Intermediate levels of expression were seen in adult colon, small intestine, testis, prostate, thymus, pancreas, kidney, skeletal muscle, placenta, and heart. Several adult tissues that express Apo-2, e.g., PBL, ovary, and spleen, have been shown previously to express DR4 [Pan et al., supra], however, the relative levels of expression of each receptor mRNA appear to be different.

EXAMPLE 8

Chromosomal Localization of the Apo-2 gene

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Chromosomal localization of the human Apo-2 gene was examined by radiation hybrid (RH) panel analysis. RH mapping was performed by PCR using a human-mouse cell radiation hybrid panel (Research Genetics) and primers based on the coding region of the Apo-2 cDNA [Gelb et al., Hum. Genet., 98:141 (1996)]. Analysis of the PCR data using the Stanford Human Genome Center Database indicates that Apo-2 is linked to the marker D8S481, with an LOD of 11.05; D8S481 is linked in turn to D8S2055, which maps to human chromosome 8p21. A similar analysis of DR4 showed that DR4 is linked to the marker D8S2127 (with an LOD of 13.00), which maps also to human chromosome 8p21.

To Applicants' present knowledge, to date, no other member of the TNFR gene family has been located to chromosome 8.

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\* \* \* \* \*

Deposit of Material

The following materials have been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, USA (ATCC):

<u>Material</u>	<u>ATCC Dep. No.</u>	<u>Deposit Date</u>
pRK5-Apo-2	_____	_____

This deposit was made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of a viable culture of the deposit for 30 years from the date of deposit. The deposit will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Genentech, Inc. and ATCC, which assures permanent and unrestricted availability of the progeny of the culture of the deposit to the public upon issuance of the pertinent U.S. patent or upon laying open to the public of any U.S. or foreign patent application, whichever comes first, and assures availability of the progeny to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC §122 and the Commissioner's rules pursuant thereto (including 37 CFR §1.14 with particular reference to 886 OG 638).

The assignee of the present application has agreed that if a culture of the materials on deposit should die or be lost or destroyed when cultivated under suitable conditions, the materials will be promptly replaced on notification with another of the same. Availability of the deposited material is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the

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invention. The present invention is not to be limited in scope by  
the construct deposited, since the deposited embodiment is intended  
as a single illustration of certain aspects of the invention and  
any constructs that are functionally equivalent are within the  
scope of this invention. The deposit of material herein does not  
constitute an admission that the written description herein  
contained is inadequate to enable the practice of any aspect of the  
invention, including the best mode thereof, nor is it to be  
construed as limiting the scope of the claims to the specific  
illustrations that it represents. Indeed, various modifications of  
the invention in addition to those shown and described herein will  
become apparent to those skilled in the art from the foregoing  
description and fall within the scope of the appended claims.

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Abstract of the Disclosure

Novel polypeptides, designated Apo-2, which are capable of modulating apoptosis are provided. Compositions including Apo-2 chimeras, nucleic acid encoding Apo-2, and antibodies to Apo-2 are also provided.

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Contact Name <u>Barbara M. Hailey</u>	

Item Name <u>DNA Plasmids</u>	
Description _____	
Quantity/Container <u>100 ng /vial</u>	# of Containers <u>25 vials</u>
Shipment Temperature: <input type="checkbox"/> Wet Ice <input type="checkbox"/> Dry Ice <input checked="" type="checkbox"/> Ambient	
Hazard Classification: <input type="checkbox"/> Radioactive <input type="checkbox"/> Flammable <input type="checkbox"/> Poison <input type="checkbox"/> Biological <input checked="" type="checkbox"/> Non-Hazardous <input type="checkbox"/> Carcinogen <input type="checkbox"/> Corrosive <input type="checkbox"/> Toxic <input type="checkbox"/> Infectious/Etiologic Agent	
Special Handling: <input type="checkbox"/> Latex Gloves <input type="checkbox"/> Safety Glasses <input type="checkbox"/> Face Mask <input checked="" type="checkbox"/> None <input type="checkbox"/> Chemical Gloves <input type="checkbox"/> Safety Goggles <input type="checkbox"/> Apron/Lab Coat <input type="checkbox"/> Other (explain)	

Item Name _____	
Description _____	
Quantity/Container _____	# of Containers _____
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Hazard Classification: <input type="checkbox"/> Radioactive <input type="checkbox"/> Flammable <input type="checkbox"/> Poison <input type="checkbox"/> Biological <input type="checkbox"/> Non-Hazardous <input type="checkbox"/> Carcinogen <input type="checkbox"/> Corrosive <input type="checkbox"/> Toxic <input type="checkbox"/> Infectious/Etiologic Agent	
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Item Name _____	
Description _____	
Quantity/Container _____	# of Containers _____
Shipment Temperature: <input type="checkbox"/> Wet Ice <input type="checkbox"/> Dry Ice <input type="checkbox"/> Ambient	
Hazard Classification: <input type="checkbox"/> Radioactive <input type="checkbox"/> Flammable <input type="checkbox"/> Poison <input type="checkbox"/> Biological <input type="checkbox"/> Non-Hazardous <input type="checkbox"/> Carcinogen <input type="checkbox"/> Corrosive <input type="checkbox"/> Toxic <input type="checkbox"/> Infectious/Etiologic Agent	
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Digestion with EcoRI and Hind III yields about a 1750 bp fragment.
7. Provide an indication of the properties of the strain which are or may be dangerous to health or the environment.  
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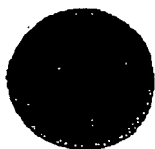
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May 13, 1997

### VIA FACSIMILE

Frank P. Simione  
Director, Professional Services  
American Type Culture Collection  
12301 Parklawn Drive  
Rockville, MD 20852-1776

Re: Deposit of pRK5-Apo-2 (Case No. P1101)

Dear Mr. Simione:

In response to your facsimile dated May 9, 1997, please be advised that the source of the Apo-2 DNA insert is human. The pRK5 plasmid was derived from *E. coli*.

Sincerely,

  
Diane L. Marschang

DLM/jt



**AMERICAN TYPE CULTURE COLLECTION**

12301 Parklawn Drive  
Rockville, Maryland 20852 USA

Telephone: 301/231-5519  
FAX: 301/816-4366

**FACSIMILE**

**Date:** May 15, 1997

**To:** Diane L. Marschang  
Genentech, Inc.

**Fax Number:** 1-415-952-9881

**Total number of pages including this page:** One (1)

**From:** ATCC Patent Depository

**Reference:** Patent Deposit on behalf of Genentech, Inc. (Ref. Case No. P1101).

**Plasmid** pRK5-Apo-2 assigned ATCC 209021.

**Date of deposit** May 8, 1997. Paperwork will be forwarded to you in a few days.

**An invoice will be sent under separate cover as follows:**

One time fee - 30 years	\$ 600.00
Informing of Requesters	360.00
Viability Test	<u>150.00</u>

**Total amount due to ATCC 209021** **\$ 1,110.00**

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*Barbara M. Hailey*  
**Barbara M. Hailey, Administrator, ATCC Patent Depository**  
**Telephone: 301/231-5519**

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# American Type Culture Collection

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## BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

### INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT ISSUED PURSUANT TO RULE 7.3  
AND VIABILITY STATEMENT ISSUED PURSUANT TO RULE 10.2

**To: (Name and Address of Depositor or Attorney)**

Genentech, Inc.  
Attn: Janet E. Hasak  
460 Point San Bruno Blvd.  
So. San Francisco, CA 94080

**Deposited on Behalf of:** Genentech, Inc. (Ref. Case No. P1101)

**Identification Reference by Depositor:**

**ATCC Designation**

Plasmid pRK5-Apo-2

209021

The deposit was accompanied by: \_\_\_ a scientific description \_ a proposed taxonomic description indicated above.

The deposit was received May 8, 1997 by this International Depository Authority and has been accepted.

**AT YOUR REQUEST:** ☒ We will inform you of requests for the strain for 30 years.

The strain will be made available if a patent office signatory to the Budapest Treaty certifies one's right to receive, or if a U.S. Patent is issued citing the strain, and ATCC is instructed by the United States Patent & Trademark Office or the depositor to release said strain.

If the culture should die or be destroyed during the effective term of the deposit, it shall be your responsibility to replace it with living culture of the same.

The strain will be maintained for a period of at least 30 years from date of deposit, or five years after the most recent request for a sample, whichever is longer. The United States and many other countries are signatory to the Budapest Treaty.

The viability of the culture cited above was tested May 15, 1997. On that date, the culture was viable.

**International Depository Authority:** American Type Culture Collection, Rockville, Md. 20852 USA

**Signature of person having authority to represent ATCC:**

Barbara M. Hailey  
Barbara M. Hailey, Administrator, Patent Depository

**Date:** May 15, 1997

**cc:** Diane L. Marschang



5

Apo-2 Receptor

FIELD OF THE INVENTION

10 The present invention relates generally to the  
identification, isolation, and recombinant production of novel  
polypeptides, designated herein as "Apo-2".

BACKGROUND OF THE INVENTION

Apoptosis or "Programmed Cell Death"

15 Control of cell numbers in mammals is believed to be  
determined, in part, by a balance between cell proliferation and  
cell death. One form of cell death, sometimes referred to as  
necrotic cell death, is typically characterized as a pathologic  
form of cell death resulting from some trauma or cellular injury.  
20 In contrast, there is another, "physiologic" form of cell death  
which usually proceeds in an orderly or controlled manner. This  
orderly or controlled form of cell death is often referred to as  
"apoptosis" [see, e.g., Barr et al., Bio/Technology, 12:487-493  
(1994); Steller et al., Science, 267:1445-1449 (1995)]. Apoptotic  
25 cell death naturally occurs in many physiological processes,  
including embryonic development and clonal selection in the immune  
system [Itoh et al., Cell, 66:233-243 (1991)]. Decreased levels of  
apoptotic cell death have been associated with a variety of  
pathological conditions, including cancer, lupus, and herpes virus  
30 infection [Thompson, Science, 267:1456-1462 (1995)]. Increased  
levels of apoptotic cell death may be associated with a variety of  
other pathological conditions, including AIDS, Alzheimer's disease,  
Parkinson's disease, amyotrophic lateral sclerosis, multiple  
sclerosis, retinitis pigmentosa, cerebellar degeneration, aplastic  
35 anemia, myocardial infarction, stroke, reperfusion injury, and  
toxin-induced liver disease [see, Thompson, supra].

Apoptotic cell death is typically accompanied by one or more characteristic morphological and biochemical changes in cells, such as condensation of cytoplasm, loss of plasma membrane microvilli, segmentation of the nucleus, degradation of chromosomal DNA or loss of mitochondrial function. A variety of extrinsic and intrinsic signals are believed to trigger or induce such morphological and biochemical cellular changes [Raff, Nature, 356:397-400 (1992); Steller, supra; Sachs et al., Blood, 82:15 (1993)]. For instance, they can be triggered by hormonal stimuli, such as glucocorticoid hormones for immature thymocytes, as well as withdrawal of certain growth factors [Watanabe-Fukunaga et al., Nature, 356:314-317 (1992)]. Also, some identified oncogenes such as *myc*, *rel*, and *ElA*, and tumor suppressors, like *p53*, have been reported to have a role in inducing apoptosis. Certain chemotherapy drugs and some forms of radiation have likewise been observed to have apoptosis-inducing activity [Thompson, supra].

#### TNF Family of Cytokines

Various molecules, such as tumor necrosis factor- $\alpha$  ("TNF- $\alpha$ "), tumor necrosis factor- $\beta$  ("TNF- $\beta$ " or "lymphotoxin"), CD30 ligand, CD27 ligand, CD40 ligand, OX-40 ligand, 4-1BB ligand, Apo-1 ligand (also referred to as Fas ligand or CD95 ligand), and Apo-2 ligand (also referred to as TRAIL) have been identified as members of the tumor necrosis factor ("TNF") family of cytokines [See, e.g., Gruss and Dower, Blood, 85:3378-3404 (1995); Wiley et al., Immunity, 3:673-682 (1995); Pitti et al., J. Biol. Chem., 271:12687-12690 (1996)]. Among these molecules, TNF- $\alpha$ , TNF- $\beta$ , CD30 ligand, 4-1BB ligand, Apo-1 ligand, and Apo-2 ligand (TRAIL) have been reported to be involved in apoptotic cell death. Both TNF- $\alpha$  and TNF- $\beta$  have been reported to induce apoptotic death in susceptible tumor cells [Schmid et al., Proc. Natl. Acad. Sci., 83:1881 (1986); Dealtry et al., Eur. J. Immunol., 17:689 (1987)]. Zheng et al. have reported that TNF- $\alpha$  is involved in post-stimulation apoptosis of CD8-positive T cells [Zheng et al., Nature, 377:348-351 (1995)]. Other investigators have reported that CD30 ligand may be involved in deletion of self-reactive T cells in the thymus [Amakawa et al., Cold Spring Harbor Laboratory

Symposium on Programmed Cell Death, Abstr. No. 10, (1995)].

5 Mutations in the mouse Fas/Apo-1 receptor or ligand genes  
(called *lpr* and *gld*, respectively) have been associated with some  
autoimmune disorders, indicating that Apo-1 ligand may play a role  
in regulating the clonal deletion of self-reactive lymphocytes in  
the periphery [Krammer et al., Curr. Op. Immunol., 6:279-289  
(1994); Nagata et al., Science, 267:1449-1456 (1995)]. Apo-1  
ligand is also reported to induce post-stimulation apoptosis in  
CD4-positive T lymphocytes and in B lymphocytes, and may be  
10 involved in the elimination of activated lymphocytes when their  
function is no longer needed [Krammer et al., supra; Nagata et al.,  
supra]. Agonist mouse monoclonal antibodies specifically binding  
to the Apo-1 receptor have been reported to exhibit cell killing  
activity that is comparable to or similar to that of TNF- $\alpha$   
15 [Yonehara et al., J. Exp. Med., 169:1747-1756 (1989)].

#### TNF Family of Receptors

Induction of various cellular responses mediated by such  
TNF family cytokines is believed to be initiated by their binding  
to specific cell receptors. Two distinct TNF receptors of  
20 approximately 55-kDa (TNFR1) and 75-kDa (TNFR2) have been  
identified [Hohman et al., J. Biol. Chem., 264:14927-14934 (1989);  
Brockhaus et al., Proc. Natl. Acad. Sci., 87:3127-3131 (1990); EP  
417,563, published March 20, 1991] and human and mouse cDNAs  
corresponding to both receptor types have been isolated and  
25 characterized [Loetscher et al., Cell, 61:351 (1990); Schall et  
al., Cell, 61:361 (1990); Smith et al., Science, 248:1019-1023  
(1990); Lewis et al., Proc. Natl. Acad. Sci., 88:2830-2834 (1991);  
Goodwin et al., Mol. Cell. Biol., 11:3020-3026 (1991)]. Extensive  
polymorphisms have been associated with both TNF receptor genes  
30 [see, e.g., Takao et al., Immunogenetics, 37:199-203 (1993)]. Both  
TNFRs share the typical structure of cell surface receptors  
including extracellular, transmembrane and intracellular regions.  
The extracellular portions of both receptors are found naturally  
also as soluble TNF-binding proteins [Nophar, Y. et al., EMBO J.,  
35 9:3269 (1990); and Kohno, T. et al., Proc. Natl. Acad. Sci. U.S.A.,

87:8331 (1990)]. More recently, the cloning of recombinant soluble TNF receptors was reported by Hale et al. [J. Cell. Biochem. Supplement 15F, 1991, p. 113 (P424)].

The extracellular portion of type 1 and type 2 TNFRs (TNFR1 and TNFR2) contains a repetitive amino acid sequence pattern of four cysteine-rich domains (CRDs) designated 1 through 4, starting from the NH<sub>2</sub>-terminus. Each CRD is about 40 amino acids long and contains 4 to 6 cysteine residues at positions which are well conserved [Schall et al., supra; Loetscher et al., supra; Smith et al., supra; Nophar et al., supra; Kohno et al., supra]. In TNFR1, the approximate boundaries of the four CRDs are as follows: CRD1- amino acids 14 to about 53; CRD2- amino acids from about 54 to about 97; CRD3- amino acids from about 98 to about 138; CRD4- amino acids from about 139 to about 167. In TNFR2, CRD1 includes amino acids 17 to about 54; CRD2- amino acids from about 55 to about 97; CRD3- amino acids from about 98 to about 140; and CRD4- amino acids from about 141 to about 179 [Banner et al., Cell, 73:431-435 (1993)]. The potential role of the CRDs in ligand binding is also described by Banner et al., supra.

A similar repetitive pattern of CRDs exists in several other cell-surface proteins, including the p75 nerve growth factor receptor (NGFR) [Johnson et al., Cell, 47:545 (1986); Radeke et al., Nature, 325:593 (1987)], the B cell antigen CD40 [Stamenkovic et al., EMBO J., 8:1403 (1989)], the T cell antigen OX40 [Mallet et al., EMBO J., 9:1063 (1990)] and the Fas antigen [Yonehara et al., supra and Itoh et al., supra]. CRDs are also found in the soluble TNFR (sTNFR)-like T2 proteins of the Shope and myxoma poxviruses [Upton et al., Virology, 160:20-29 (1987); Smith et al., Biochem. Biophys. Res. Commun., 176:335 (1991); Upton et al., Virology, 184:370 (1991)]. Optimal alignment of these sequences indicates that the positions of the cysteine residues are well conserved. These receptors are sometimes collectively referred to as members of the TNF/NGF receptor superfamily. Recent studies on p75NGFR showed that the deletion of CRD1 [Welcher, A.A. et al., Proc. Natl. Acad. Sci. USA, 88:159-163 (1991)] or a 5-amino acid insertion in

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this domain [Yan, H. and Chao, M.V., J. Biol. Chem., 266:12099-12104 (1991)] had little or no effect on NGF binding [Yan, H. and Chao, M.V., supra]. p75 NGFR contains a proline-rich stretch of about 60 amino acids, between its CRD4 and transmembrane region, which is not involved in NGF binding [Peetre, C. et al., Eur. J. Hematol., 41:414-419 (1988); Seckinger, P. et al., J. Biol. Chem., 264:11966-11973 (1989); Yan, H. and Chao, M.V., supra]. A similar proline-rich region is found in TNFR2 but not in TNFR1.

10 Itoh et al. disclose that the Apo-1 receptor can signal an apoptotic cell death similar to that signaled by the 55-kDa TNFR1 [Itoh et al., supra]. Expression of the Apo-1 antigen has also been reported to be down-regulated along with that of TNFR1 when cells are treated with either TNF- $\alpha$  or anti-Apo-1 mouse monoclonal antibody [Krammer et al., supra; Nagata et al., supra]. Accordingly, some investigators have hypothesized that cell lines that co-express both Apo-1 and TNFR1 receptors may mediate cell killing through common signaling pathways [Id.].

20 The TNF family ligands identified to date, with the exception of lymphotoxin- $\alpha$ , are type II transmembrane proteins, whose C-terminus is extracellular. In contrast, the receptors in the TNF receptor (TNFR) family identified to date are type I transmembrane proteins. In both the TNF ligand and receptor families, however, homology identified between family members has been found mainly in the extracellular domain ("ECD"). Several of the TNF family cytokines, including TNF- $\alpha$ , Apo-1 ligand and CD40 ligand, are cleaved proteolytically at the cell surface; the resulting protein in each case typically forms a homotrimeric molecule that functions as a soluble cytokine. TNF receptor family proteins are also usually cleaved proteolytically to release soluble receptor ECDs that can function as inhibitors of the cognate cytokines.

30 Recently, other members of the TNFR family have been identified. In Marsters et al., Curr. Biol., 6:750 (1996), investigators describe a full length native sequence human polypeptide, called Apo-3, which exhibits similarity to the TNFR

family in its extracellular cysteine-rich repeats and resembles TNFR1 and CD95 in that it contains a cytoplasmic death domain sequence [see also Marsters et al., Curr. Biol., 6:1669 (1996)]. Apo-3 has also been referred to by other investigators as DR3, wsl-1 and TRAMP [Chinnaiyan et al., Science, 274:990 (1996); Kitson et al., Nature, 384:372 (1996); Bodmer et al., Immunity, 6:79 (1997)].

Pan et al. have disclosed another TNF receptor family member referred to as "DR4" [Pan et al., Science, 276:111-113 (1997)]. The DR4 was reported to contain a cytoplasmic death domain capable of engaging the cell suicide apparatus. Pan et al. disclose that DR4 is believed to be a receptor for the ligand known as Apo-2 ligand or TRAIL.

#### The Apoptosis-Inducing Signaling Complex

As presently understood, the cell death program contains at least three important elements - activators, inhibitors, and effectors; in *C. elegans*, these elements are encoded respectively by three genes, *Ced-4*, *Ced-9* and *Ced-3* [Steller, Science, 267:1445 (1995); Chinnaiyan et al., Science, 275:1122-1126 (1997)]. Two of the TNFR family members, TNFR1 and Fas/Apo1 (CD95), can activate apoptotic cell death [Chinnaiyan and Dixit, Current Biology, 6:555-562 (1996); Fraser and Evan, Cell, 85:781-784 (1996)]. TNFR1 is also known to mediate activation of the transcription factor, NF- $\kappa$ B [Tartaglia et al., Cell, 74:845-853 (1993); Hsu et al., Cell, 84:299-308 (1996)]. In addition to some ECD homology, these two receptors share homology in their intracellular domain (ICD) in an oligomerization interface known as the death domain [Tartaglia et al., supra; Nagata, Cell, 88:355 (1997)]. Death domains are also found in several metazoan proteins that regulate apoptosis, namely, the *Drosophila* protein, Reaper, and the mammalian proteins referred to as FADD/MORT1, TRADD, and RIP [Cleaveland and Ihle, Cell, 81:479-482 (1995)]. Using the yeast-two hybrid system, Raven et al. report the identification of protein, wsl-1, which binds to the TNFR1 death domain [Raven et al., Programmed Cell Death Meeting, September 20-24, 1995, Abstract at page 127; Raven et al., European Cytokine Network, 7:Abstr. 82 at page 210 (April-June 1996)]. The

wsl-1 protein is described as being homologous to TNFR1 (48% identity) and having a restricted tissue distribution. According to Raven et al., the tissue distribution of wsl-1 is significantly different from the TNFR1 binding protein, TRADD.

5           Upon ligand binding and receptor clustering, TNFR1 and CD95 are believed to recruit FADD into a death-inducing signalling complex. CD95 purportedly binds FADD directly, while TNFR1 binds FADD indirectly via TRADD [Chinnaiyan et al., Cell, 81:505-512 (1995); Boldin et al., J. Biol. Chem., 270:387-391 (1995); Hsu et al., supra; Chinnaiyan et al., J. Biol. Chem., 271:4961-4965 (1996)]. It has been reported that FADD serves as an adaptor protein which recruits the Ced-3-related protease, MACH $\alpha$ /FLICE (caspase 8), into the death signalling complex [Boldin et al., Cell, 85:803-815 (1996); Muzio et al., Cell, 85:817-827 (1996)]. MACH $\alpha$ /FLICE appears to be the trigger that sets off a cascade of apoptotic proteases, including the interleukin-1 $\beta$  converting enzyme (ICE) and CPP32/Yama, which may execute some critical aspects of the cell death programme [Fraser and Evan, supra].

15           It was recently disclosed that programmed cell death involves the activity of members of a family of cysteine proteases related to the *C. elegans* cell death gene, *ced-3*, and to the mammalian IL-1-converting enzyme, ICE. The activity of the ICE and CPP32/Yama proteases can be inhibited by the product of the cowpox virus gene, *crmA* [Ray et al., Cell, 69:597-604 (1992); Tewari et al., Cell, 81:801-809 (1995)]. Recent studies show that CrmA can inhibit TNFR1- and CD95-induced cell death [Enari et al., Nature, 375:78-81 (1995); Tewari et al., J. Biol. Chem., 270:3255-3260 (1995)].

20           As reviewed recently by Tewari et al., TNFR1, TNFR2 and CD40 modulate the expression of proinflammatory and costimulatory cytokines, cytokine receptors, and cell adhesion molecules through activation of the transcription factor, NF- $\kappa$ B [Tewari et al., Curr. Op. Genet. Develop., 6:39-44 (1996)]. NF- $\kappa$ B is the prototype of a family of dimeric transcription factors whose subunits contain conserved Rel regions [Verma et al., Genes Develop., 9:2723-2735

(1996); Baldwin, Ann. Rev. Immunol., 14:649-681 (1996)]. In its latent form, NF- $\kappa$ B is complexed with members of the I $\kappa$ B inhibitor family; upon inactivation of the I $\kappa$ B in response to certain stimuli, released NF- $\kappa$ B translocates to the nucleus where it binds to specific DNA sequences and activates gene transcription.

For a review of the TNF family of cytokines and their receptors, see Gruss and Dower, supra.

#### SUMMARY OF THE INVENTION

Applicants have identified cDNA clones that encode novel polypeptides, designated in the present application as "Apo-2." It is believed that Apo-2 is a member of the TNFR family; full-length native sequence human Apo-2 polypeptide exhibits some similarities to some known TNFRs, including a cytoplasmic death domain region. Full-length native sequence human Apo-2 also exhibits similarity to the TNFR family in its extracellular cysteine-rich repeats. Apo-2 polypeptide has been found to be capable of triggering caspase-dependent apoptosis and activating NF- $\kappa$ B. Applicants surprisingly found that the soluble extracellular domain of Apo-2 binds Apo-2 ligand (Apo-2L) and can inhibit Apo-2 ligand function. It is presently believed that Apo-2 ligand can signal via at least two different receptors, DR4 and the newly described Apo-2 herein.

In one embodiment, the invention provides isolated Apo-2 polypeptide. In particular, the invention provides isolated native sequence Apo-2 polypeptide, which in one embodiment, includes an amino acid sequence comprising residues 1 to 411 of Figure 1 (SEQ ID NO:1). In other embodiments, the isolated Apo-2 polypeptide comprises at least about 80% amino acid sequence identity with native sequence Apo-2 polypeptide comprising residues 1 to 411 of Figure 1 (SEQ ID NO:1).

In another embodiment, the invention provides an isolated extracellular domain (ECD) sequence of Apo-2. Optionally, the isolated extracellular domain sequence comprises amino acid residues 54 to 182 of Fig. 1 (SEQ ID NO:1).

In another embodiment, the invention provides an isolated



death domain sequence of Apo-2. Optionally, the isolated death domain sequence comprises amino acid residues 324 to 391 of Fig. 1 (SEQ ID NO:1).

In another embodiment, the invention provides chimeric molecules comprising Apo-2 polypeptide fused to a heterologous polypeptide or amino acid sequence. An example of such a chimeric molecule comprises an Apo-2 fused to an immunoglobulin sequence. Another example comprises an extracellular domain sequence of Apo-2 fused to a heterologous polypeptide or amino acid sequence, such as an immunoglobulin sequence.

In another embodiment, the invention provides an isolated nucleic acid molecule encoding Apo-2 polypeptide. In one aspect, the nucleic acid molecule is RNA or DNA that encodes an Apo-2 polypeptide or a particular domain of Apo-2, or is complementary to such encoding nucleic acid sequence, and remains stably bound to it under at least moderate, and optionally, under high stringency conditions. In one embodiment, the nucleic acid sequence is selected from:

(a) the coding region of the nucleic acid sequence of Figure 1 (SEQ ID NO:2) that codes for residue 1 to residue 411 (i.e., nucleotides 140-142 through 1370-1372), inclusive;

(b) the coding region of the nucleic acid sequence of Figure 1 (SEQ ID NO:2) that codes for residue 1 to residue 182 (i.e., nucleotides 140-142 through 683-685), inclusive;

(c) the coding region of the nucleic acid sequence of Figure 1 (SEQ ID NO:2) that codes for residue 54 to residue 182 (i.e., nucleotides 299-301 through 683-685), inclusive;

(d) the coding region of the nucleic acid sequence of Figure 1 (SEQ ID NO:2) that codes for residue 324 to residue 391 (i.e., nucleotides 1109-1111 through 1310-1312), inclusive; or

(e) a sequence corresponding to the sequence of (a), (b), (c) or (d) within the scope of degeneracy of the genetic code.

In a further embodiment, the invention provides a vector comprising the nucleic acid molecule encoding the Apo-2 polypeptide or particular domain of Apo-2. A host cell comprising the vector

or the nucleic acid molecule is also provided. A method of producing Apo-2 is further provided.

In another embodiment, the invention provides an antibody which specifically binds to Apo-2. The antibody may be an agonistic, antagonistic or neutralizing antibody.

In another embodiment, the invention provides non-human, transgenic or knock-out animals.

A further embodiment of the invention provides articles of manufacture and kits that include Apo-2 or Apo-2 antibodies.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the nucleotide sequence of a native sequence human Apo-2 cDNA and its derived amino acid sequence.

Figure 2A shows the derived amino acid sequence of a native sequence human Apo-2 - the putative signal sequence is underlined, the putative transmembrane domain is boxed, and the putative death domain sequence is dash underlined. The cysteines of the two cysteine-rich domains are individually underlined.

Figure 2B shows an alignment and comparison of the death domain sequences of native sequence human Apo-2, DR4, Apo-3/DR3, TNFR1, and Fas/Apo-1 (CD95). Asterisks indicate residues that are essential for death signaling by TNFR1 [Tartaglia et al., *supra*].

Figure 3 shows the interaction of the Apo-2 ECD with Apo-2L. Supernatants from mock-transfected 293 cells or from 293 cells transfected with Flag epitope-tagged Apo-2 ECD were incubated with poly-His-tagged Apo-2L and subjected to immunoprecipitation with anti-Flag conjugated or Nickel conjugated agarose beads. The precipitated proteins were resolved by electrophoresis on polyacrylamide gels, and detected by immunoblot with anti-Apo-2L or anti-Flag antibody.

Figure 4 shows the induction of apoptosis by Apo-2 and inhibition of Apo-2L activity by soluble Apo-2 ECD. Human 293 cells (A, B) or HeLa cells (C) were transfected by pRK5 vector or by pRK5-based plasmids encoding Apo-2 and/or CrmA. Apoptosis was assessed by morphology (A), DNA fragmentation (B), or by FACS (C-

E). Soluble Apo-2L was pre-incubated with buffer or affinity-purified Apo-2 ECD together with anti-Flag antibody or Apo-2 ECD immunoaderhin or DR4 or TNFR1 immunoaderhins and added to HeLa cells. The cells were later analyzed for apoptosis (D). Dose-response analysis using Apo-2L with Apo-2 ECD immunoaderhin was also determined (E).

Figure 5 shows activation of NF- $\kappa$ B by Apo-2, DR4, and Apo-2L. (A) HeLa cells were transfected with expression plasmids encoding the indicated proteins. Nuclear extracts were prepared and analyzed by an electrophoretic mobility shift assay. (B) HeLa cells or MCF7 cells were treated with buffer, Apo-2L or TNF-alpha and assayed for NF- $\kappa$ B activity. (C) HeLa cells were preincubated with buffer, ALLN or cyclohexamide before addition of Apo-2L. Apoptosis was later analyzed by FACS.

Figure 6 shows expression of Apo-2 mRNA in human tissues as analyzed by Northern hybridization of human tissue poly A RNA blots.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

##### I. Definitions

The terms "Apo-2 polypeptide" and "Apo-2" when used herein encompass native sequence Apo-2 and Apo-2 variants (which are further defined herein). These terms encompass Apo-2 from a variety of mammals, including humans. The Apo-2 may be isolated from a variety of sources, such as from human tissue types or from another source, or prepared by recombinant or synthetic methods.

A "native sequence Apo-2" comprises a polypeptide having the same amino acid sequence as an Apo-2 derived from nature. Thus, a native sequence Apo-2 can have the amino acid sequence of naturally-occurring Apo-2 from any mammal. Such native sequence Apo-2 can be isolated from nature or can be produced by recombinant or synthetic means. The term "native sequence Apo-2" specifically encompasses naturally-occurring truncated or secreted forms of the Apo-2 (e.g., an extracellular domain sequence), naturally-occurring variant forms (e.g., alternatively spliced forms) and naturally-

occurring allelic variants of the Apo-2. A naturally-occurring variant form of the Apo-2 includes an Apo-2 having an amino acid substitution at residue 410 in the amino acid sequence shown in Figure 1 (SEQ ID NO:1). In one embodiment of such naturally-occurring variant form, the leucine residue at position 410 is substituted by a methionine residue. In Fig. 1 (SEQ ID NO:1), the amino acid residue at position 410 is identified as "Xaa" to indicate that the amino acid may, optionally, be either leucine or methionine. In Fig. 1 (SEQ ID NO:2), the nucleotide at position 1367 is identified as "W" to indicate that the nucleotide may be either adenine (A) or thymine (T) or uracil (U). In one embodiment of the invention, the native sequence Apo-2 is a mature or full-length native sequence Apo-2 comprising amino acids 1 to 411 of Fig. 1 (SEQ ID NO:1).

The "Apo-2 extracellular domain" or "Apo-2 ECD" refers to a form of Apo-2 which is essentially free of the transmembrane and cytoplasmic domains of Apo-2. Ordinarily, Apo-2 ECD will have less than 1% of such transmembrane and/or cytoplasmic domains and preferably, will have less than 0.5% of such domains. Optionally, Apo-2 ECD will comprise amino acid residues 54 to 182 of Fig. 1 (SEQ ID NO:1) or amino acid residues 1 to 182 of Fig. 1 (SEQ ID NO:1).

"Apo-2 variant" means a biologically active Apo-2 as defined below having at least about 80% amino acid sequence identity with the Apo-2 having the deduced amino acid sequence shown in Fig. 1 (SEQ ID NO:1) for a full-length native sequence human Apo-2. Such Apo-2 variants include, for instance, Apo-2 polypeptides wherein one or more amino acid residues are added, or deleted, at the N- or C-terminus of the sequence of Fig. 1 (SEQ ID NO:1). Ordinarily, an Apo-2 variant will have at least about 80% amino acid sequence identity, more preferably at least about 90% amino acid sequence identity, and even more preferably at least about 95% amino acid sequence identity with the amino acid sequence of Fig. 1 (SEQ ID NO:1).

"Percent (%) amino acid sequence identity" with respect

to the Apo-2 sequences identified herein is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the Apo-2 sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as ALIGN™ or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared.

The term "epitope tagged" when used herein refers to a chimeric polypeptide comprising Apo-2, or a domain sequence thereof, fused to a "tag polypeptide". The tag polypeptide has enough residues to provide an epitope against which an antibody can be made, yet is short enough such that it does not interfere with activity of the Apo-2. The tag polypeptide preferably also is fairly unique so that the antibody does not substantially cross-react with other epitopes. Suitable tag polypeptides generally have at least six amino acid residues and usually between about 8 to about 50 amino acid residues (preferably, between about 10 to about 20 residues).

"Isolated," when used to describe the various polypeptides disclosed herein, means polypeptide that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would typically interfere with diagnostic or therapeutic uses for the polypeptide, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In preferred embodiments, the polypeptide will be purified (1) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning

cup sequenator, or (2) to homogeneity by SDS-PAGE under non-reducing or reducing conditions using Coomassie blue or, preferably, silver stain. Isolated polypeptide includes polypeptide *in situ* within recombinant cells, since at least one component of the Apo-2 natural environment will not be present. Ordinarily, however, isolated polypeptide will be prepared by at least one purification step.

An "isolated" Apo-2 nucleic acid molecule is a nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the Apo-2 nucleic acid. An isolated Apo-2 nucleic acid molecule is other than in the form or setting in which it is found in nature. Isolated Apo-2 nucleic acid molecules therefore are distinguished from the Apo-2 nucleic acid molecule as it exists in natural cells. However, an isolated Apo-2 nucleic acid molecule includes Apo-2 nucleic acid molecules contained in cells that ordinarily express Apo-2 where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that

the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

The term "antibody" is used in the broadest sense and specifically covers single anti-Apo-2 monoclonal antibodies (including agonist, antagonist, and neutralizing antibodies) and anti-Apo-2 antibody compositions with polyepitopic specificity.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally-occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen.

The monoclonal antibodies herein include hybrid and recombinant antibodies produced by splicing a variable (including hypervariable) domain of an anti-Apo-2 antibody with a constant domain (e.g. "humanized" antibodies), or a light chain with a heavy chain, or a chain from one species with a chain from another species, or fusions with heterologous proteins, regardless of species of origin or immunoglobulin class or subclass designation, as well as antibody fragments (e.g., Fab, F(ab')<sub>2</sub>, and Fv), so long as they exhibit the desired biological activity. See, e.g. U.S. Pat. No. 4,816,567 and Mage et al., in Monoclonal Antibody Production Techniques and Applications, pp.79-97 (Marcel Dekker, Inc.: New York, 1987).

Thus, the modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous

population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler and Milstein, Nature, 256:495 (1975), or may be made by recombinant DNA methods such as described in U.S. Pat. No. 4,816,567. The "monoclonal antibodies" may also be isolated from phage libraries generated using the techniques described in McCafferty et al., Nature, 348:552-554 (1990), for example.

"Humanized" forms of non-human (e.g. murine) antibodies are specific chimeric immunoglobulins, immunoglobulin chains, or fragments thereof (such as Fv, Fab, Fab', F(ab')<sub>2</sub> or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat, or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, the humanized antibody may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region or domain (Fc), typically that of a human immunoglobulin.

"Biologically active" and "desired biological activity" for the purposes herein mean having the ability to modulate



apoptosis (either in an agonistic or stimulating manner or in an antagonistic or blocking manner) in at least one type of mammalian cell *in vivo* or *ex vivo*.

5 The terms "apoptosis" and "apoptotic activity" are used in a broad sense and refer to the orderly or controlled form of cell death in mammals that is typically accompanied by one or more characteristic cell changes, including condensation of cytoplasm, loss of plasma membrane microvilli, segmentation of the nucleus, degradation of chromosomal DNA or loss of mitochondrial function.  
10 This activity can be determined and measured, for instance, by cell viability assays, FACS analysis or DNA electrophoresis, all of which are known in the art.

15 The terms "treating," "treatment," and "therapy" as used herein refer to curative therapy, prophylactic therapy, and preventative therapy.

20 The term "mammal" as used herein refers to any mammal classified as a mammal, including humans, cows, horses, dogs and cats. In a preferred embodiment of the invention, the mammal is a human.

## 25 II. Compositions and Methods of the Invention

The present invention provides newly identified and isolated Apo-2 polypeptides. In particular, Applicants have identified and isolated various human Apo-2 polypeptides. The properties and characteristics of some of these Apo-2 polypeptides are described in further detail in the Examples below. Based upon the properties and characteristics of the Apo-2 polypeptides disclosed herein, it is Applicants' present belief that Apo-2 is a member of the TNFR family.

30 A description follows as to how Apo-2, as well as Apo-2 chimeric molecules and anti-Apo-2 antibodies, may be prepared.

A. Preparation of Apo-2

The description below relates primarily to production of Apo-2 by culturing cells transformed or transfected with a vector containing Apo-2 nucleic acid. It is of course, contemplated that alternative methods, which are well known in the art, may be employed to prepare Apo-2.

1. Isolation of DNA Encoding Apo-2

The DNA encoding Apo-2 may be obtained from any cDNA library prepared from tissue believed to possess the Apo-2 mRNA and to express it at a detectable level. Accordingly, human Apo-2 DNA can be conveniently obtained from a cDNA library prepared from human tissues, such as the bacteriophage libraries of human pancreas and kidney cDNA described in Example 1. The Apo-2-encoding gene may also be obtained from a genomic library or by oligonucleotide synthesis.

Libraries can be screened with probes (such as antibodies to the Apo-2 or oligonucleotides of at least about 20-80 bases) designed to identify the gene of interest or the protein encoded by it. Screening the cDNA or genomic library with the selected probe may be conducted using standard procedures, such as described in Sambrook et al., Molecular Cloning: A Laboratory Manual (New York: Cold Spring Harbor Laboratory Press, 1989). An alternative means to isolate the gene encoding Apo-2 is to use PCR methodology [Sambrook et al., supra; Dieffenbach et al., PCR Primer: A Laboratory Manual (Cold Spring Harbor Laboratory Press, 1995)].

A preferred method of screening employs selected oligonucleotide sequences to screen cDNA libraries from various human tissues. Example 1 below describes techniques for screening a cDNA library. The oligonucleotide sequences selected as probes should be of sufficient length and sufficiently unambiguous that false positives are minimized. The oligonucleotide is preferably labeled such that it can be detected upon hybridization to DNA in the library being screened. Methods of labeling are well known in the art, and include the use of radiolabels like <sup>32</sup>P-labeled ATP, biotinylation or enzyme labeling. Hybridization conditions,

including moderate stringency and high stringency, are provided in Sambrook et al., supra.

Nucleic acid having all the protein coding sequence may be obtained by screening selected cDNA or genomic libraries using the deduced amino acid sequence disclosed herein for the first time, and, if necessary, using conventional primer extension procedures as described in Sambrook et al., supra, to detect precursors and processing intermediates of mRNA that may not have been reverse-transcribed into cDNA.

Apo-2 variants can be prepared by introducing appropriate nucleotide changes into the Apo-2 DNA, or by synthesis of the desired Apo-2 polypeptide. Those skilled in the art will appreciate that amino acid changes may alter post-translational processes of the Apo-2, such as changing the number or position of glycosylation sites or altering the membrane anchoring characteristics.

Variations in the native full-length sequence Apo-2 or in various domains of the Apo-2 described herein, can be made, for example, using any of the techniques and guidelines for conservative and non-conservative mutations set forth, for instance, in U.S. Pat. No. 5,364,934. Variations may be a substitution, deletion or insertion of one or more codons encoding the Apo-2 that results in a change in the amino acid sequence of the Apo-2 as compared with the native sequence Apo-2. Optionally the variation is by substitution of at least one amino acid with any other amino acid in one or more of the domains of the Apo-2 molecule. The variations can be made using methods known in the art such as oligonucleotide-mediated (site-directed) mutagenesis, alanine scanning, and PCR mutagenesis. Site-directed mutagenesis [Carter et al., Nucl. Acids Res., 13:4331 (1986); Zoller et al., Nucl. Acids Res., 10:6487 (1987)], cassette mutagenesis [Wells et al., Gene, 34:315 (1985)], restriction selection mutagenesis [Wells et al., Philos. Trans. R. Soc. London SerA, 317:415 (1986)] or other known techniques can be performed on the cloned DNA to produce the Apo-2 variant DNA.

Scanning amino acid analysis can also be employed to identify one or more amino acids along a contiguous sequence which are involved in the interaction with a particular ligand or receptor. Among the preferred scanning amino acids are relatively small, neutral amino acids. Such amino acids include alanine, glycine, serine, and cysteine. Alanine is the preferred scanning amino acid among this group because it eliminates the side-chain beyond the beta-carbon and is less likely to alter the main-chain conformation of the variant. Alanine is also preferred because it is the most common amino acid. Further, it is frequently found in both buried and exposed positions [Creighton, The Proteins, (W.H. Freeman & Co., N.Y.); Chothia, J. Mol. Biol., 150:1 (1976)]. If alanine substitution does not yield adequate amounts of variant, an isoteric amino acid can be used.

Once selected Apo-2 variants are produced, they can be contacted with, for instance, Apo-2L, and the interaction, if any, can be determined. The interaction between the Apo-2 variant and Apo-2L can be measured by an *in vitro* assay, such as described in the Examples below. While any number of analytical measurements can be used to compare activities and properties between a native sequence Apo-2 and an Apo-2 variant, a convenient one for binding is the dissociation constant  $K_d$  of the complex formed between the Apo-2 variant and Apo-2L as compared to the  $K_d$  for the native sequence Apo-2. Generally, a  $\geq 3$ -fold increase or decrease in  $K_d$  per substituted residue indicates that the substituted residue(s) is active in the interaction of the native sequence Apo-2 with the Apo-2L.

Optionally, representative sites in the Apo-2 sequence suitable for mutagenesis would include sites within the extracellular domain, and particularly, within one or both of the cysteine-rich domains. Such variations can be accomplished using the methods described above.

## 2. Insertion of Nucleic Acid into A Replicable Vector

The nucleic acid (e.g., cDNA or genomic DNA) encoding Apo-2 may be inserted into a replicable vector for further cloning

(amplification of the DNA) or for expression. Various vectors are publicly available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence, each of which is described below.

(i) Signal Sequence Component

The Apo-2 may be produced recombinantly not only directly, but also as a fusion polypeptide with a heterologous polypeptide, which may be a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. In general, the signal sequence may be a component of the vector, or it may be a part of the Apo-2 DNA that is inserted into the vector. The heterologous signal sequence selected preferably is one that is recognized and processed (*i.e.*, cleaved by a signal peptidase) by the host cell. The signal sequence may be a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion the signal sequence may be, *e.g.*, the yeast invertase leader, alpha factor leader (including *Saccharomyces* and *Kluyveromyces*  $\alpha$ -factor leaders, the latter described in U.S. Pat. No. 5,010,182), or acid phosphatase leader, the *C. albicans* glucoamylase leader (EP 362,179 published 4 April 1990), or the signal described in WO 90/13646 published 15 November 1990. In mammalian cell expression the native Apo-2 presequence that normally directs insertion of Apo-2 in the cell membrane of human cells *in vivo* is satisfactory, although other mammalian signal sequences may be used to direct secretion of the protein, such as signal sequences from secreted polypeptides of the same or related species, as well as viral secretory leaders, for example, the herpes simplex glycoprotein D signal.

The DNA for such precursor region is preferably ligated in reading frame to DNA encoding Apo-2.

### (ii) Origin of Replication Component

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2 $\mu$  plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used because it contains the early promoter).

Most expression vectors are "shuttle" vectors, i.e., they are capable of replication in at least one class of organisms but can be transfected into another organism for expression. For example, a vector is cloned in *E. coli* and then the same vector is transfected into yeast or mammalian cells for expression even though it is not capable of replicating independently of the host cell chromosome.

DNA may also be amplified by insertion into the host genome. This is readily accomplished using *Bacillus* species as hosts, for example, by including in the vector a DNA sequence that is complementary to a sequence found in *Bacillus* genomic DNA. Transfection of *Bacillus* with this vector results in homologous recombination with the genome and insertion of Apo-2 DNA. However, the recovery of genomic DNA encoding Apo-2 is more complex than that of an exogenously replicated vector because restriction enzyme digestion is required to excise the Apo-2 DNA.

### (iii) Selection Gene Component

Expression and cloning vectors typically contain a selection gene, also termed a selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host

cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g., the gene encoding D-alanine racemase for *Bacilli*.

One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene produce a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin [Southern et al., J. Molec. Appl. Genet., 1:327 (1982)], mycophenolic acid (Mulligan et al., Science, 209:1422 (1980)) or hygromycin [Sugden et al., Mol. Cell. Biol., 5:410-413 (1985)]. The three examples given above employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug G418 or neomycin (geneticin), xgpt (mycophenolic acid), or hygromycin, respectively.

Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the Apo-2 nucleic acid, such as DHFR or thymidine kinase. The mammalian cell transformants are placed under selection pressure that only the transformants are uniquely adapted to survive by virtue of having taken up the marker. Selection pressure is imposed by culturing the transformants under conditions in which the concentration of selection agent in the medium is successively changed, thereby leading to amplification of both the selection gene and the DNA that encodes Apo-2. Amplification is the process by which genes in greater demand for the production of a protein critical for growth are reiterated in tandem within the chromosomes of successive generations of recombinant cells. Increased quantities of Apo-2 are synthesized from the amplified DNA. Other examples of amplifiable genes include metallothionein-I and -II, adenosine deaminase, and

ornithine decarboxylase.

Cells transformed with the DHFR selection gene may first be identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub et al., Proc. Natl. Acad. Sci. USA, 77:4216 (1980). The transformed cells are then exposed to increased levels of methotrexate. This leads to the synthesis of multiple copies of the DHFR gene, and, concomitantly, multiple copies of other DNA comprising the expression vectors, such as the DNA encoding Apo-2. This amplification technique can be used with any otherwise suitable host, e.g., ATCC No. CCL61 CHO-K1, notwithstanding the presence of endogenous DHFR if, for example, a mutant DHFR gene that is highly resistant to Mtx is employed (EP 117,060).

Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding Apo-2, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3'-phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, e.g., kanamycin, neomycin, or G418. See U.S. Patent No. 4,965,199.

A suitable selection gene for use in yeast is the *trp1* gene present in the yeast plasmid YRp7 [Stinchcomb et al., Nature, 282:39 (1979); Kingsman et al., Gene, 7:141 (1979); Tschemper et al., Gene, 10:157 (1980)]. The *trp1* gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1 [Jones, Genetics, 85:12 (1977)]. The presence of the *trp1* lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, *Leu2*-deficient yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the *Leu2* gene.



In addition, vectors derived from the 1.6  $\mu$ m circular plasmid pKD1 can be used for transformation of *Kluyveromyces* yeasts [Bianchi et al., Curr. Genet., 12:185 (1987)]. More recently, an expression system for large-scale production of recombinant calf chymosin was reported for *K. lactis* [Van den Berg, Bio/Technology, 8:135 (1990)]. Stable multi-copy expression vectors for secretion of mature recombinant human serum albumin by industrial strains of *Kluyveromyces* have also been disclosed [Fleer et al., Bio/Technology, 9:968-975 (1991)].

(iv) Promoter Component

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the Apo-2 nucleic acid sequence. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of particular nucleic acid sequence, such as the Apo-2 nucleic acid sequence, to which they are operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, e.g., the presence or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These promoters are operably linked to Apo-2 encoding DNA by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector. Both the native Apo-2 promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of the Apo-2 DNA.

Promoters suitable for use with prokaryotic hosts include the  $\beta$ -lactamase and lactose promoter systems [Chang et al., Nature, 275:615 (1978); Goeddel et al., Nature, 281:544 (1979)], alkaline phosphatase, a tryptophan (trp) promoter system [Goeddel, Nucleic Acids Res., 8:4057 (1980); EP 36,776], and hybrid promoters such as the tac promoter [deBoer et al., Proc. Natl. Acad. Sci. USA, 80:21-

25 (1983)]. However, other known bacterial promoters are suitable. Their nucleotide sequences have been published, thereby enabling a skilled worker operably to ligate them to DNA encoding Apo-2 [Siebenlist et al., Cell, 20:269 (1980)] using linkers or adaptors to supply any required restriction sites. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding Apo-2.

Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CXCAAT region where X may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into eukaryotic expression vectors.

Examples of suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase [Hitzeman et al., J. Biol. Chem., 255:2073 (1980)] or other glycolytic enzymes [Hess et al., J. Adv. Enzyme Reg., 7:149 (1968); Holland, Biochemistry, 17:4900 (1978)], such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytichrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in EP 73,657. Yeast enhancers also are advantageously used with yeast promoters.

Apo-2 transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published 5 July 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40), from heterologous mammalian promoters, e.g., the actin promoter or an immunoglobulin promoter, from heat-shock promoters, and from the promoter normally associated with the Apo-2 sequence, provided such promoters are compatible with the host cell systems.

The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication [Fiers et al., Nature, 273:113 (1978); Mulligan and Berg, Science, 209:1422-1427 (1980); Pavlakis et al., Proc. Natl. Acad. Sci. USA, 78:7398-7402 (1981)]. The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment [Greenaway et al., Gene, 18:355-360 (1982)]. A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. Patent No. 4,419,446. A modification of this system is described in U.S. Patent No. 4,601,978 [See also Gray et al., Nature, 295:503-508 (1982) on expressing cDNA encoding immune interferon in monkey cells; Reyes et al., Nature, 297:598-601 (1982) on expression of human  $\beta$ -interferon cDNA in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus; Canaani and Berg, Proc. Natl. Acad. Sci. USA 79:5166-5170 (1982) on expression of the human interferon  $\beta$ 1 gene in cultured mouse and rabbit cells; and Gorman et al., Proc. Natl. Acad. Sci. USA, 79:6777-6781 (1982) on expression of bacterial CAT sequences in CV-1 monkey kidney cells, chicken embryo fibroblasts, Chinese hamster ovary cells, HeLa cells, and mouse NIH-3T3 cells using the Rous sarcoma virus long terminal repeat as a promoter].

(v) Enhancer Element Component

Transcription of a DNA encoding the Apo-2 of this

invention by higher eukaryotes may be increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp, that act on a promoter to increase its transcription. Enhancers are relatively orientation and position independent, having been found 5' [Laimins et al., Proc. Natl. Acad. Sci. USA, 78:993 (1981)] and 3' [Lusky et al., Mol. Cell Bio., 3:1108 (1983)] to the transcription unit, within an intron [Banerji et al., Cell, 33:729 (1983)], as well as within the coding sequence itself [Osborne et al., Mol. Cell Bio., 4:1293 (1984)]. Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin,  $\alpha$ -fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, Nature, 297:17-18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to the Apo-2 coding sequence, but is preferably located at a site 5' from the promoter.

#### (vi) Transcription Termination Component

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding Apo-2.

#### (vii) Construction and Analysis of Vectors

Construction of suitable vectors containing one or more of the above-listed components employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and re-ligated in the form desired to generate the

plasmids required.

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures can be used to transform *E. coli* K12 strain 294 (ATCC 31,446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced by the method of Messing et al., Nucleic Acids Res., 9:309 (1981) or by the method of Maxam et al., Methods in Enzymology, 65:499 (1980).

(viii) Transient Expression Vectors

Expression vectors that provide for the transient expression in mammalian cells of DNA encoding Apo-2 may be employed. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector [Sambrook et al., *supra*]. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of such polypeptides for desired biological or physiological properties. Thus, transient expression systems are particularly useful in the invention for purposes of identifying Apo-2 variants.

(ix) Suitable Exemplary Vertebrate Cell Vectors

Other methods, vectors, and host cells suitable for adaptation to the synthesis of Apo-2 in recombinant vertebrate cell culture are described in Gething et al., Nature, 293:620-625 (1981); Mantei et al., Nature, 281:40-46 (1979); EP 117,060; and EP 117,058.

3. Selection and Transformation of Host Cells

Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include but are not limited to eubacteria, such as Gram-negative or

Gram-positive organisms, for example, Enterobacteriaceae such as *Escherichia*, e.g., *E. coli*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Proteus*, *Salmonella*, e.g., *Salmonella typhimurium*, *Serratia*, e.g., *Serratia marcescans*, and *Shigella*, as well as Bacilli such as *B. subtilis* and *B. licheniformis* (e.g., *B. licheniformis* 41P disclosed in DD 266,710 published 12 April 1989), *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*. Preferably, the host cell should secrete minimal amounts of proteolytic enzymes.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for Apo-2-encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein.

Suitable host cells for the expression of glycosylated Apo-2 are derived from multicellular organisms. Such host cells are capable of complex processing and glycosylation activities. In principle, any higher eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified [See, e.g., Luckow et al., Bio/Technology, 6:47-55 (1988); Miller et al., in Genetic Engineering, Setlow et al., eds., Vol. 8 (Plenum Publishing, 1986), pp. 277-279; and Maeda et al., Nature, 315:592-594 (1985)]. A variety of viral strains for transfection are publicly available, e.g., the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV.

Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can be utilized as hosts. Typically, plant cells are transfected by incubation with certain strains of the bacterium *Agrobacterium tumefaciens*. During incubation of the plant cell culture with *A. tumefaciens*, the DNA encoding the Apo-2

can be transferred to the plant cell host such that it is transfected, and will, under appropriate conditions, express the Apo-2-encoding DNA. In addition, regulatory and signal sequences compatible with plant cells are available, such as the nopaline synthase promoter and polyadenylation signal sequences [Depicker et al., J. Mol. Appl. Gen., 1:561 (1982)]. In addition, DNA segments isolated from the upstream region of the T-DNA 780 gene are capable of activating or increasing transcription levels of plant-expressible genes in recombinant DNA-containing plant tissue [EP 321,196 published 21 June 1989].

Propagation of vertebrate cells in culture (tissue culture) is also well known in the art [See, e.g., Tissue Culture, Academic Press, Kruse and Patterson, editors (1973)]. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., J. Gen Virol., 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, Proc. Natl. Acad. Sci. USA, 77:4216 (1980)); mouse sertoli cells (TM4, Mather, Biol. Reprod., 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., Annals N.Y. Acad. Sci., 383:44-68 (1982)); MRC 5 cells; and FS4 cells.

Host cells are transfected and preferably transformed with the above-described expression or cloning vectors for Apo-2 production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in

fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example,  $\text{CaPO}_4$  and electroporation. Successful transfection is generally recognized when any indication of the operation of this vector occurs within the host cell.

5 Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as  
10 described in Sambrook et al., supra, or electroporation is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. Infection with *Agrobacterium tumefaciens* is used for transformation of certain plant cells, as described by Shaw et al., Gene, 23:315 (1983) and WO 89/05859 published 29 June 1989. In addition, plants may be transfected using ultrasound treatment as described in WO 91/00358 published 10  
15 January 1991.

For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb, Virology, 52:456-457 (1978) is preferred. General aspects of mammalian cell  
20 host system transformations have been described in U.S. Pat. No. 4,399,216. Transformations into yeast are typically carried out according to the method of Van Solingen et al., J. Bact., 130:946 (1977) and Hsiao et al., Proc. Natl. Acad. Sci. (USA), 76:3829 (1979). However, other methods for introducing DNA into cells, such as by nuclear microinjection, electroporation, bacterial protoplast fusion with intact cells, or polycations, e.g., polybrene, polyornithine, may also be used. For various techniques for transforming mammalian cells, see Keown et al., Methods in  
25 Enzymology, 185:527-537 (1990) and Mansour et al., Nature, 336:348-352 (1988).

#### 4. Culturing the Host Cells

Prokaryotic cells used to produce Apo-2 may be cultured in suitable media as described generally in Sambrook et al., supra.

35 The mammalian host cells used to produce Apo-2 may be



cultured in a variety of media. Examples of commercially available media include Ham's F10 (Sigma), Minimal Essential Medium ("MEM", Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ("DMEM", Sigma). Any such media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

In general, principles, protocols, and practical techniques for maximizing the productivity of mammalian cell cultures can be found in Mammalian Cell Biotechnology: a Practical Approach, M. Butler, ed. (IRL Press, 1991).

The host cells referred to in this disclosure encompass cells in culture as well as cells that are within a host animal.

#### 5. Detecting Gene Amplification/Expression

Gene amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA [Thomas, Proc. Natl. Acad. Sci. USA, 77:5201-5205 (1980)], dot blotting (DNA analysis), or *in situ* hybridization, using an appropriately labeled probe, based on the sequences provided herein. Various labels may be employed, most commonly radioisotopes, and particularly <sup>32</sup>P. However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionucleotides, fluorescers or enzymes. Alternatively,

antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of cells or tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. With immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and fixation, followed by reaction with labeled antibodies specific for the gene product coupled, where the labels are usually visually detectable, such as enzymatic labels, fluorescent labels, or luminescent labels.

Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared in any mammal. Conveniently, the antibodies may be prepared against a native sequence Apo-2 polypeptide or against a synthetic peptide based on the DNA sequences provided herein or against exogenous sequence fused to Apo-2 DNA and encoding a specific antibody epitope.

#### 6. Purification of Apo-2 Polypeptide

Forms of Apo-2 may be recovered from culture medium or from host cell lysates. If the Apo-2 is membrane-bound, it can be released from the membrane using a suitable detergent solution (e.g. Triton-X 100) or its extracellular domain may be released by enzymatic cleavage.

When Apo-2 is produced in a recombinant cell other than one of human origin, the Apo-2 is free of proteins or polypeptides of human origin. However, it may be desired to purify Apo-2 from recombinant cell proteins or polypeptides to obtain preparations that are substantially homogeneous as to Apo-2. As a first step, the culture medium or lysate may be centrifuged to remove particulate cell debris. Apo-2 thereafter is purified from

contaminant soluble proteins and polypeptides, with the following procedures being exemplary of suitable purification procedures: by fractionation on an ion-exchange column; ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; and protein A Sepharose columns to remove contaminants such as IgG.

Apo-2 variants in which residues have been deleted, inserted, or substituted can be recovered in the same fashion as native sequence Apo-2, taking account of changes in properties occasioned by the variation. For example, preparation of an Apo-2 fusion with another protein or polypeptide, e.g., a bacterial or viral antigen, immunoglobulin sequence, or receptor sequence, may facilitate purification; an immunoaffinity column containing antibody to the sequence can be used to adsorb the fusion polypeptide. Other types of affinity matrices also can be used.

A protease inhibitor such as phenyl methyl sulfonyl fluoride (PMSF) also may be useful to inhibit proteolytic degradation during purification, and antibiotics may be included to prevent the growth of adventitious contaminants. One skilled in the art will appreciate that purification methods suitable for native sequence Apo-2 may require modification to account for changes in the character of Apo-2 or its variants upon expression in recombinant cell culture.

#### 7. Covalent Modifications of Apo-2 Polypeptides

Covalent modifications of Apo-2 are included within the scope of this invention. One type of covalent modification of the Apo-2 is introduced into the molecule by reacting targeted amino acid residues of the Apo-2 with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues of the Apo-2.

Derivatization with bifunctional agents is useful for crosslinking Apo-2 to a water-insoluble support matrix or surface for use in the method for purifying anti-Apo-2 antibodies, and

vice-versa. Derivatization with one or more bifunctional agents will also be useful for crosslinking Apo-2 molecules to generate Apo-2 dimers. Such dimers may increase binding avidity and extend half-life of the molecule in vivo. Commonly used crosslinking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidyl-propionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)-dithio]propioimide yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Patent Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

Other modifications include deamidation of glutamyl and asparaginyl residues to the corresponding glutamyl and aspartyl residues, respectively, hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the  $\alpha$ -amino groups of lysine, arginine, and histidine side chains [T.E. Creighton, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)], acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group. The modified forms of the residues fall within the scope of the present invention.

Another type of covalent modification of the Apo-2 polypeptide included within the scope of this invention comprises altering the native glycosylation pattern of the polypeptide. "Altering the native glycosylation pattern" is intended for purposes herein to mean deleting one or more carbohydrate moieties found in native sequence Apo-2, and/or adding one or more glycosylation sites that are not present in the native sequence Apo-2.

Glycosylation of polypeptides is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-acetylgalactosamine, galactose, or xylose to a hydroxylamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Addition of glycosylation sites to the Apo-2 polypeptide may be accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the native sequence Apo-2 (for O-linked glycosylation sites). The Apo-2 amino acid sequence may optionally be altered through changes at the DNA level, particularly by mutating the DNA encoding the Apo-2 polypeptide at preselected bases such that codons are generated that will translate into the desired amino acids. The DNA mutation(s) may be made using methods described above and in U.S. Pat. No. 5,364,934, *supra*.

Another means of increasing the number of carbohydrate moieties on the Apo-2 polypeptide is by chemical or enzymatic coupling of glycosides to the polypeptide. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. These methods are described in WO 87/05330 published 11 September 1987, and in Aplin and Wriston, CRC Crit. Rev. Biochem., pp. 259-306 (1981).

Removal of carbohydrate moieties present on the Apo-2 polypeptide may be accomplished chemically or enzymatically or by mutational substitution of codons encoding for amino acid residues that serve as targets for glycosylation. For instance, chemical deglycosylation by exposing the polypeptide to the compound trifluoromethanesulfonic acid, or an equivalent compound can result in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the polypeptide intact. Chemical deglycosylation is described by Hakimuddin, et al., Arch. Biochem. Biophys., 259:52 (1987) and by Edge et al., Anal. Biochem., 118:131 (1981). Enzymatic cleavage of carbohydrate moieties on polypeptides can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura et al., Meth. Enzymol., 138:350 (1987).

Glycosylation at potential glycosylation sites may be prevented by the use of the compound tunicamycin as described by Duskin et al., J. Biol. Chem., 257:3105 (1982). Tunicamycin blocks the formation of protein-N-glycoside linkages.

Another type of covalent modification of Apo-2 comprises linking the Apo-2 polypeptide to one of a variety of nonproteinaceous polymers, e.g., polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

#### 8. Apo-2 Chimeras

The present invention also provides chimeric molecules comprising Apo-2 fused to another, heterologous polypeptide or amino acid sequence.

In one embodiment, the chimeric molecule comprises a fusion of the Apo-2 with a tag polypeptide which provides an epitope to which an anti-tag antibody can selectively bind. The epitope tag is generally placed at the amino- or carboxyl- terminus of the Apo-2. The presence of such epitope-tagged forms of the Apo-2 can be detected using an antibody against the tag polypeptide. Also, provision of the epitope tag enables the Apo-2

to be readily purified by affinity purification using an anti-tag antibody or another type of affinity matrix that binds to the epitope tag.

Various tag polypeptides and their respective antibodies are well known in the art. Examples include the flu HA tag polypeptide and its antibody 12CA5 [Field et al., Mol. Cell. Biol., 8:2159-2165 (1988)]; the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto [Evan et al., Molecular and Cellular Biology, 5:3610-3616 (1985)]; and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody [Paborsky et al., Protein Engineering, 3(6):547-553 (1990)]. Other tag polypeptides include the Flag-peptide [Hopp et al., BioTechnology, 6:1204-1210 (1988)]; the KT3 epitope peptide [Martin et al., Science, 255:192-194 (1992)]; an  $\alpha$ -tubulin epitope peptide [Skinner et al., J. Biol. Chem., 266:15163-15166 (1991)]; and the T7 gene 10 protein peptide tag [Lutz-Freyermuth et al., Proc. Natl. Acad. Sci. USA, 87:6393-6397 (1990)]. Once the tag polypeptide has been selected, an antibody thereto can be generated using the techniques disclosed herein.

Generally, epitope-tagged Apo-2 may be constructed and produced according to the methods described above. Epitope-tagged Apo-2 is also described in the Examples below. Apo-2-tag polypeptide fusions are preferably constructed by fusing the cDNA sequence encoding the Apo-2 portion in-frame to the tag polypeptide DNA sequence and expressing the resultant DNA fusion construct in appropriate host cells. Ordinarily, when preparing the Apo-2-tag polypeptide chimeras of the present invention, nucleic acid encoding the Apo-2 will be fused at its 3' end to nucleic acid encoding the N-terminus of the tag polypeptide, however 5' fusions are also possible. For example, a polyhistidine sequence of about 5 to about 10 histidine residues may be fused at the N-terminus or the C-terminus and used as a purification handle in affinity chromatography.

Epitope-tagged Apo-2 can be purified by affinity chromatography using the anti-tag antibody. The matrix to which

the affinity antibody is attached may include, for instance, agarose, controlled pore glass or poly(styrenedivinyl)benzene. The epitope-tagged Apo-2 can then be eluted from the affinity column using techniques known in the art.

5           In another embodiment, the chimeric molecule comprises an Apo-2 polypeptide fused to an immunoglobulin sequence. The chimeric molecule may also comprise a particular domain sequence of Apo-2, such as the extracellular domain sequence of native Apo-2 fused to an immunoglobulin sequence. This includes chimeras in  
10 monomeric, homo- or heteromultimeric, and particularly homo- or heterodimeric, or -tetrameric forms; optionally, the chimeras may be in dimeric forms or homodimeric heavy chain forms. Generally, these assembled immunoglobulins will have known unit structures as represented by the following diagrams.



X or A  $\backslash$   $\text{C}_H$  or  $\text{C}_L$

5

X or A  $\backslash$   $\text{Y}$   $\text{C}_H$  or  $\text{C}_L$

10

A  $\backslash$   $\text{C}_L$   
A  $\backslash$   $\text{C}_H$

15

A  $\backslash$   $\text{C}_L$   
 $\text{V}_H$   $\backslash$   $\text{C}_H$

20

$\text{V}_L$   $\backslash$   $\text{C}_L$   
A  $\backslash$   $\text{C}_H$

25

X  $\backslash$   $\text{C}_L$   
A  $\backslash$   $\text{C}_H$

30

A  $\backslash$   $\text{C}_L$   
X  $\backslash$   $\text{C}_H$

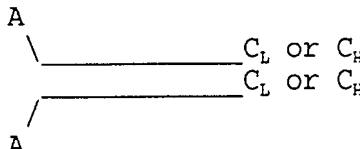
35

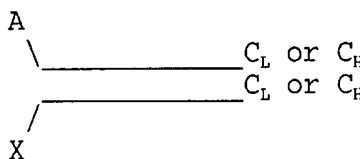
A basic four chain structural unit is the form in which IgG, IgD, and IgE exist. A four chain unit is repeated in the higher molecular weight immunoglobulins; IgM generally exists as a pentamer of basic four-chain units held together by disulfide bonds. IgA globulin, and occasionally IgG globulin, may also exist in a multimeric form in serum. In the case of multimers, each four chain unit may be the same or different.

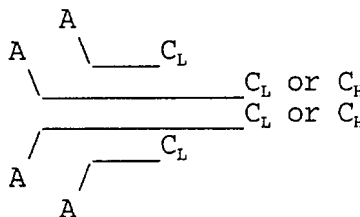
The following diagrams depict some exemplary monomer, homo- and heterodimer and homo- and heteromultimer structures. These diagrams are merely illustrative, and the chains of the

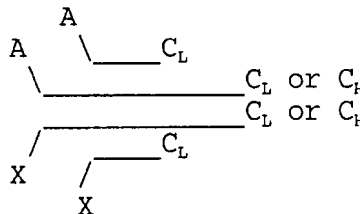
multimers are believed to be disulfide bonded in the same fashion as native immunoglobulins.

monomer: A \_\_\_\_\_ C<sub>L</sub> or C<sub>H</sub>

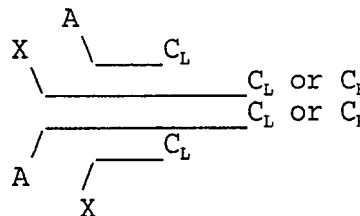
homodimer: 

heterodimer: 

homotetramer: 

heterotetramer: 

and



In the foregoing diagrams, "A" means an Apo-2 sequence or an Apo-2 sequence fused to a heterologous sequence; X is an additional agent, which may be the same as A or different, a

portion of an immunoglobulin superfamily member such as a variable region or a variable region-like domain, including a native or chimeric immunoglobulin variable region, a toxin such a pseudomonas exotoxin or ricin, or a sequence functionally binding to another protein, such as other cytokines (i.e., IL-1, interferon- $\gamma$ ) or cell surface molecules (i.e., NGFR, CD40, OX40, Fas antigen, T2 proteins of Shope and myxoma poxviruses), or a polypeptide therapeutic agent not otherwise normally associated with a constant domain; Y is a linker or another receptor sequence; and  $V_L$ ,  $V_H$ ,  $C_L$  and  $C_H$  represent light or heavy chain variable or constant domains of an immunoglobulin. Structures comprising at least one CRD of an Apo-2 sequence as "A" and another cell-surface protein having a repetitive pattern of CRDs (such as TNFR) as "X" are specifically included.

It will be understood that the above diagrams are merely exemplary of the possible structures of the chimeras of the present invention, and do not encompass all possibilities. For example, there might desirably be several different "A"s, "X"s, or "Y"s in any of these constructs. Also, the heavy or light chain constant domains may be originated from the same or different immunoglobulins. All possible permutations of the illustrated and similar structures are all within the scope of the invention herein.

In general, the chimeric molecules can be constructed in a fashion similar to chimeric antibodies in which a variable domain from an antibody of one species is substituted for the variable domain of another species. See, for example, EP 0 125 023; EP 173,494; Munro, Nature, 312:597 (13 December 1984); Neuberger et al., Nature, 312:604-608 (13 December 1984); Sharon et al., Nature, 309:364-367 (24 May 1984); Morrison et al., Proc. Nat'l. Acad. Sci. USA, 81:6851-6855 (1984); Morrison et al., Science, 229:1202-1207 (1985); Boulianne et al., Nature, 312:643-646 (13 December 1984); Capon et al., Nature, 337:525-531 (1989); Traunecker et al., Nature, 339:68-70 (1989).

Alternatively, the chimeric molecules may be constructed

as follows. The DNA including a region encoding the desired sequence, such as an Apo-2 and/or TNFR sequence, is cleaved by a restriction enzyme at or proximal to the 3' end of the DNA encoding the immunoglobulin-like domain(s) and at a point at or near the DNA encoding the N-terminal end of the Apo-2 or TNFR polypeptide (where use of a different leader is contemplated) or at or proximal to the N-terminal coding region for TNFR (where the native signal is employed). This DNA fragment then is readily inserted proximal to DNA encoding an immunoglobulin light or heavy chain constant region and, if necessary, the resulting construct tailored by deletional mutagenesis. Preferably, the Ig is a human immunoglobulin when the chimeric molecule is intended for *in vivo* therapy for humans. DNA encoding immunoglobulin light or heavy chain constant regions is known or readily available from cDNA libraries or is synthesized. See for example, Adams et al., Biochemistry, 19:2711-2719 (1980); Gough et al., Biochemistry, 19:2702-2710 (1980); Dolby et al., Proc. Natl. Acad. Sci. USA, 77:6027-6031 (1980); Rice et al., Proc. Natl. Acad. Sci., 79:7862-7865 (1982); Falkner et al., Nature, 298:286-288 (1982); and Morrison et al., Ann. Rev. Immunol., 2:239-256 (1984).

Further details of how to prepare such fusions are found in publications concerning the preparation of immunoadhesins. Immunoadhesins in general, and CD4-Ig fusion molecules specifically are disclosed in WO 89/02922, published 6 April 1989). Molecules comprising the extracellular portion of CD4, the receptor for human immunodeficiency virus (HIV), linked to IgG heavy chain constant region are known in the art and have been found to have a markedly longer half-life and lower clearance than the soluble extracellular portion of CD4 [Capon et al., *supra*; Byrn et al., Nature, 344:667 (1990)]. The construction of specific chimeric TNFR-IgG molecules is also described in Ashkenazi et al. Proc. Natl. Acad. Sci., 88:10535-10539 (1991); Lesslauer et al. [J. Cell. Biochem. Supplement 15F, 1991, p. 115 (P 432)]; and Peppel and Beutler, J. Cell. Biochem. Supplement 15F, 1991, p. 118 (P 439)].

B. Therapeutic and Non-therapeutic Uses for Apo-2

Apo-2, as disclosed in the present specification, can be employed therapeutically to induce apoptosis in mammalian cells. This therapy can be accomplished for instance, using *in vivo* or *ex vivo* gene therapy techniques and includes the use of the death domain sequences disclosed herein. The Apo-2 chimeric molecules (including the chimeric molecules containing the extracellular domain sequence of Apo-2) comprising immunoglobulin sequences can also be employed therapeutically to inhibit apoptosis or NF- $\kappa$ B induction by Apo-2L or by another ligand that Apo-2 binds to.

The Apo-2 of the invention also has utility in non-therapeutic applications. Nucleic acid sequences encoding the Apo-2 may be used as a diagnostic for tissue-specific typing. For example, procedures like *in situ* hybridization, Northern and Southern blotting, and PCR analysis may be used to determine whether DNA and/or RNA encoding Apo-2 is present in the cell type(s) being evaluated. Apo-2 nucleic acid will also be useful for the preparation of Apo-2 by the recombinant techniques described herein.

The isolated Apo-2 may be used in quantitative diagnostic assays as a control against which samples containing unknown quantities of Apo-2 may be prepared. Apo-2 preparations are also useful in generating antibodies, as standards in assays for Apo-2 (e.g., by labeling Apo-2 for use as a standard in a radioimmunoassay, radioreceptor assay, or enzyme-linked immunoassay), in affinity purification techniques, and in competitive-type receptor binding assays when labeled with, for instance, radioiodine, enzymes, or fluorophores.

Modified forms of the Apo-2, such as the Apo-2-IgG chimeric molecules (immunoadhesins) described above, can be used as immunogens in producing anti-Apo-2 antibodies.

Nucleic acids which encode Apo-2 or its modified forms can also be used to generate either transgenic animals or "knock out" animals which, in turn, are useful in the development and screening of therapeutically useful reagents. A transgenic animal

(e.g., a mouse or rat) is an animal having cells that contain a transgene, which transgene was introduced into the animal or an ancestor of the animal at a prenatal, e.g., an embryonic stage. A transgene is a DNA which is integrated into the genome of a cell from which a transgenic animal develops. In one embodiment, cDNA encoding Apo-2 or an appropriate sequence thereof (such as Apo-2-IgG) can be used to clone genomic DNA encoding Apo-2 in accordance with established techniques and the genomic sequences used to generate transgenic animals that contain cells which express DNA encoding Apo-2. Methods for generating transgenic animals, particularly animals such as mice or rats, have become conventional in the art and are described, for example, in U.S. Patent Nos. 4,736,866 and 4,870,009. Typically, particular cells would be targeted for Apo-2 transgene incorporation with tissue-specific enhancers. Transgenic animals that include a copy of a transgene encoding Apo-2 introduced into the germ line of the animal at an embryonic stage can be used to examine the effect of increased expression of DNA encoding Apo-2. Such animals can be used as tester animals for reagents thought to confer protection from, for example, pathological conditions associated with excessive apoptosis. In accordance with this facet of the invention, an animal is treated with the reagent and a reduced incidence of the pathological condition, compared to untreated animals bearing the transgene, would indicate a potential therapeutic intervention for the pathological condition. In another embodiment, transgenic animals that carry a soluble form of Apo-2 such as the Apo-2 ECD or an immunoglobulin chimera of such form could be constructed to test the effect of chronic neutralization of Apo-2L, a ligand of Apo-2.

Alternatively, non-human homologues of Apo-2 can be used to construct an Apo-2 "knock out" animal which has a defective or altered gene encoding Apo-2 as a result of homologous recombination between the endogenous gene encoding Apo-2 and altered genomic DNA encoding Apo-2 introduced into an embryonic cell of the animal. For example, cDNA encoding Apo-2 can be used to clone genomic DNA encoding Apo-2 in accordance with established techniques. A

portion of the genomic DNA encoding Apo-2 can be deleted or replaced with another gene, such as a gene encoding a selectable marker which can be used to monitor integration. Typically, several kilobases of unaltered flanking DNA (both at the 5' and 3' ends) are included in the vector [see e.g., Thomas and Capecchi, Cell, 51:503 (1987) for a description of homologous recombination vectors]. The vector is introduced into an embryonic stem cell line (e.g., by electroporation) and cells in which the introduced DNA has homologously recombined with the endogenous DNA are selected [see e.g., Li et al., Cell, 69:915 (1992)]. The selected cells are then injected into a blastocyst of an animal (e.g., a mouse or rat) to form aggregation chimeras [see e.g., Bradley, in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E. J. Robertson, ed. (IRL, Oxford, 1987), pp. 113-152]. A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term to create a "knock out" animal. Progeny harboring the homologously recombined DNA in their germ cells can be identified by standard techniques and used to breed animals in which all cells of the animal contain the homologously recombined DNA. Knockout animals can be characterized for instance, for their ability to defend against certain pathological conditions and for their development of pathological conditions due to absence of the Apo-2 polypeptide, including for example, development of tumors.

C. Anti-Apo-2 Antibody Preparation

The present invention further provides anti-Apo-2 antibodies. Antibodies against Apo-2 may be prepared as follows. Exemplary antibodies include polyclonal, monoclonal, humanized, bispecific, and heteroconjugate antibodies.

1. Polyclonal Antibodies

The Apo-2 antibodies may comprise polyclonal antibodies. Methods of preparing polyclonal antibodies are known to the skilled artisan. Polyclonal antibodies can be raised in a mammal, for example, by one or more injections of an immunizing agent and, if desired, an adjuvant. Typically, the immunizing agent and/or

adjuvant will be injected in the mammal by multiple subcutaneous or intraperitoneal injections. The immunizing agent may include the Apo-2 polypeptide or a fusion protein thereof. An example of a suitable immunizing agent is a Apo-2-IgG fusion protein or chimeric molecule (including an Apo-2 ECD-IgG fusion protein). Cells expressing Apo-2 at their surface may also be employed. It may be useful to conjugate the immunizing agent to a protein known to be immunogenic in the mammal being immunized. Examples of such immunogenic proteins which may be employed include but are not limited to keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, and soybean trypsin inhibitor. An aggregating agent such as alum may also be employed to enhance the mammal's immune response. Examples of adjuvants which may be employed include Freund's complete adjuvant and MPL-TDM adjuvant (monophosphoryl Lipid A, synthetic trehalose dicorynomycolate). The immunization protocol may be selected by one skilled in the art without undue experimentation. The mammal can then be bled, and the serum assayed for antibody titer. If desired, the mammal can be boosted until the antibody titer increases or plateaus.

## 2. Monoclonal Antibodies

The Apo-2 antibodies may, alternatively, be monoclonal antibodies. Monoclonal antibodies may be prepared using hybridoma methods, such as those described by Kohler and Milstein, supra. In a hybridoma method, a mouse, hamster, or other appropriate host animal, is typically immunized (such as described above) with an immunizing agent to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the immunizing agent. Alternatively, the lymphocytes may be immunized *in vitro*.

The immunizing agent will typically include the Apo-2 polypeptide or a fusion protein thereof. An example of a suitable immunizing agent is a Apo-2-IgG fusion protein or chimeric molecule. Cells expressing Apo-2 at their surface may also be employed. Generally, either peripheral blood lymphocytes ("PBLs") are used if cells of human origin are desired, or spleen cells or



lymph node cells are used if non-human mammalian sources are desired. The lymphocytes are then fused with an immortalized cell line using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell [Goding, Monoclonal Antibodies: Principles and Practice, Academic Press, (1986) pp. 59-103]. Immortalized cell lines are usually transformed mammalian cells, particularly myeloma cells of rodent, bovine and human origin. Usually, rat or mouse myeloma cell lines are employed. The hybridoma cells may be cultured in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, immortalized cells. For example, if the parental cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine ("HAT medium"), which substances prevent the growth of HGPRT-deficient cells.

Preferred immortalized cell lines are those that fuse efficiently, support stable high level expression of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. More preferred immortalized cell lines are murine myeloma lines, which can be obtained, for instance, from the Salk Institute Cell Distribution Center, San Diego, California and the American Type Culture Collection, Rockville, Maryland. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies [Kozbor, J. Immunol., 133:3001 (1984); Brodeur et al., Monoclonal Antibody Production Techniques and Applications, Marcel Dekker, Inc., New York, (1987) pp. 51-63].

The culture medium in which the hybridoma cells are cultured can then be assayed for the presence of monoclonal antibodies directed against Apo-2. Preferably, the binding specificity of monoclonal antibodies produced by the hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA). Such techniques and assays are

known in the art. The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson and Pollard, Anal. Biochem., 107:220 (1980).

After the desired hybridoma cells are identified, the clones may be subcloned by limiting dilution procedures and grown by standard methods [Goding, supra]. Suitable culture media for this purpose include, for example, Dulbecco's Modified Eagle's Medium and RPMI-1640 medium. Alternatively, the hybridoma cells may be grown *in vivo* as ascites in a mammal.

The monoclonal antibodies secreted by the subclones may be isolated or purified from the culture medium or ascites fluid by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

The monoclonal antibodies may also be made by recombinant DNA methods, such as those described in U.S. Patent No. 4,816,567. DNA encoding the monoclonal antibodies of the invention can be readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells of the invention serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. The DNA also may be modified, for example, by substituting the coding sequence for human heavy and light chain constant domains in place of the homologous murine sequences [U.S. Patent No. 4,816,567; Morrison et al., supra] or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. Such a non-immunoglobulin polypeptide can be substituted for the constant domains of an antibody of the invention, or can be substituted for the variable domains of one antigen-combining site of an antibody

of the invention to create a chimeric bivalent antibody.

The antibodies may be monovalent antibodies. Methods for preparing monovalent antibodies are well known in the art. For example, one method involves recombinant expression of immunoglobulin light chain and modified heavy chain. The heavy chain is truncated generally at any point in the Fc region so as to prevent heavy chain crosslinking. Alternatively, the relevant cysteine residues are substituted with another amino acid residue or are deleted so as to prevent crosslinking.

In vitro methods are also suitable for preparing monovalent antibodies. Digestion of antibodies to produce fragments thereof, particularly, Fab fragments, can be accomplished using routine techniques known in the art. For instance, digestion can be performed using papain. Examples of papain digestion are described in WO 94/29348 published 12/22/94 and U.S. Patent No. 4,342,566. Papain digestion of antibodies typically produces two identical antigen binding fragments, called Fab fragments, each with a single antigen binding site, and a residual Fc fragment. Pepsin treatment yields an F(ab')<sub>2</sub> fragment that has two antigen combining sites and is still capable of cross-linking antigen.

The Fab fragments produced in the antibody digestion also contain the constant domains of the light chain and the first constant domain (CH<sub>1</sub>) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH<sub>1</sub> domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. F(ab')<sub>2</sub> antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

### 3. Humanized Antibodies

The Apo-2 antibodies of the invention may further comprise humanized antibodies or human antibodies. Humanized forms of non-human (e.g., murine) antibodies are chimeric

immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab')<sub>2</sub> or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin [Jones et al., Nature, 321:522-525 (1986); Reichmann et al., Nature, 332:323-329 (1988); and Presta, Curr. Op. Struct. Biol., 2:593-596 (1992)].

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers [Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature, 332:323-327 (1988); Verhoeven et al., Science, 239:1534-1536 (1988)], by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Patent No. 4,816,567),

wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important in order to reduce antigenicity. According to the "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody [Sims et al., J. Immunol., 151:2296 (1993); Chothia and Lesk, J. Mol. Biol., 196:901 (1987)]. Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies [Carter et al., Proc. Natl. Acad. Sci. USA, 89:4285 (1992); Presta et al., J. Immunol., 151:2623 (1993)].

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three dimensional models of the parental and humanized sequences. Three dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can

be selected and combined from the consensus and import sequence so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding [see, WO 94/04679 published 3 March 1994].

Transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production can be employed. For example, it has been described that the homozygous deletion of the antibody heavy chain joining region ( $J_H$ ) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge [see, e.g., Jakobovits et al., Proc. Natl. Acad. Sci. USA, 90:2551-255 (1993); Jakobovits et al., Nature, 362:255-258 (1993); Bruggermann et al., Year in Immuno., 7:33 (1993)]. Human antibodies can also be produced in phage display libraries [Hoogenboom and Winter, J. Mol. Biol., 227:381 (1991); Marks et al., J. Mol. Biol., 222:581 (1991)]. The techniques of Cote et al. and Boerner et al. are also available for the preparation of human monoclonal antibodies (Cote et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985) and Boerner et al., J. Immunol., 147(1):86-95 (1991)].

#### 4. Bispecific Antibodies

Bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens. In the present case, one of the binding specificities is for the Apo-2, the other one is for any other antigen, and preferably for a cell-surface protein or receptor or receptor subunit.

Methods for making bispecific antibodies are known in the art. Traditionally, the recombinant production of bispecific antibodies is based on the co-expression of two immunoglobulin heavy-chain/light-chain pairs, where the two heavy chains have

different specificities [Millstein and Cuello, Nature, 305:537-539 (1983)]. Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of ten different antibody molecules, of which only one has the correct bispecific structure. The purification of the correct molecule is usually accomplished by affinity chromatography steps. Similar procedures are disclosed in WO 93/08829, published 13 May 1993, and in Traunecker et al., EMBO J., 10:3655-3659 (1991).

According to a different and more preferred approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy-chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light-chain binding present in at least one of the fusions. DNAs encoding the immunoglobulin heavy-chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance. In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy-chain/light-chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in

only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690 published 3 March 1994. For further details of generating bispecific antibodies see, for example, Suresh et al., Methods in Enzymology, 121:210 (1986).

#### 5. Heteroconjugate Antibodies

Heteroconjugate antibodies are also within the scope of the present invention. Heteroconjugate antibodies are composed of two covalently joined antibodies. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells [US Patent No. 4,676,980], and for treatment of HIV infection [WO 91/00360; WO 92/200373; EP 03089]. It is contemplated that the antibodies may be prepared in vitro using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins may be constructed using a disulfide exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptobutyrimidate and those disclosed, for example, in U.S. Pat. No. 4,676,980.

#### D. Therapeutic and Non-therapeutic Uses for Apo-2 Antibodies

The Apo-2 antibodies of the invention have therapeutic utility. Agonistic Apo-2 antibodies, for instance, may be employed to activate or stimulate apoptosis in cancer cells. Alternatively, antagonistic antibodies may be used to block excessive apoptosis (for instance in neurodegenerative disease) or to block potential autoimmune/inflammatory effects of Apo-2 resulting from NF- $\kappa$ B activation.

Apo-2 antibodies may further be used in diagnostic assays for Apo-2, e.g., detecting its expression in specific cells, tissues, or serum. Various diagnostic assay techniques known in the art may be used, such as competitive binding assays, direct or indirect sandwich assays and immunoprecipitation assays conducted in either heterogeneous or homogeneous phases [Zola, Monoclonal Antibodies: A Manual of Techniques, CRC Press, Inc. (1987) pp. 147-158]. The antibodies used in the diagnostic assays can be labeled



with a detectable moiety. The detectable moiety should be capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety may be a radioisotope, such as  $^3\text{H}$ ,  $^{14}\text{C}$ ,  $^{32}\text{P}$ ,  $^{35}\text{S}$ , or  $^{125}\text{I}$ , a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin, or an enzyme, such as alkaline phosphatase, beta-galactosidase or horseradish peroxidase. Any method known in the art for conjugating the antibody to the detectable moiety may be employed, including those methods described by Hunter et al., Nature, 144:945 (1962); David et al., Biochemistry, 13:1014 (1974); Pain et al., J. Immunol. Meth., 40:219 (1981); and Nygren, J. Histochem. and Cytochem., 30:407 (1982).

Apo-2 antibodies also are useful for the affinity purification of Apo-2 from recombinant cell culture or natural sources. In this process, the antibodies against Apo-2 are immobilized on a suitable support, such a Sephadex resin or filter paper, using methods well known in the art. The immobilized antibody then is contacted with a sample containing the Apo-2 to be purified, and thereafter the support is washed with a suitable solvent that will remove substantially all the material in the sample except the Apo-2, which is bound to the immobilized antibody. Finally, the support is washed with another suitable solvent that will release the Apo-2 from the antibody.

#### E. Kits Containing Apo-2 or Apo-2 Antibodies

In a further embodiment of the invention, there are provided articles of manufacture and kits containing Apo-2 or Apo-2 antibodies which can be used, for instance, for the therapeutic or non-therapeutic applications described above. The article of manufacture comprises a container with a label. Suitable containers include, for example, bottles, vials, and test tubes. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which includes an active agent that is effective for therapeutic or non-therapeutic applications, such as described above. The active agent in the composition is Apo-2 or an Apo-2 antibody. The label

on the container indicates that the composition is used for a specific therapy or non-therapeutic application, and may also indicate directions for either *in vivo* or *in vitro* use, such as those described above.

5           The kit of the invention will typically comprise the container described above and one or more other containers comprising materials desirable from a commercial and user standpoint, including buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

10           \*\*\*\*\*

The following examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

15           All patent and literature references cited in the present specification are hereby incorporated by reference in their entirety.

#### EXAMPLES

20           All restriction enzymes referred to in the examples were purchased from New England Biolabs and used according to manufacturer's instructions. All other commercially available reagents referred to in the examples were used according to manufacturer's instructions unless otherwise indicated. The source of those cells identified in the following examples, and throughout the specification, by ATCC accession numbers is the American Type Culture Collection, Rockville, Maryland.

#### EXAMPLE 1

##### Isolation of cDNA clones Encoding Human Apo-2

30           Expressed sequence tag (EST) DNA databases (LIFESEQ™, Incyte Pharmaceuticals, Palo Alto, CA) were searched and an EST was identified which showed homology to the death domain of the Apo-3 receptor [Marsters et al., Curr. Biol., 6:750 (1996)]. Human pancreas and kidney lgt10 bacteriophage cDNA libraries (both  
35           purchased from Clontech) were ligated into pRK5 vectors as follows.

Reagents were added together and incubated at 16°C for 16 hours: 5X  
T4 ligase buffer (3 ml); pRK5, Xho1, Not1 digested vector, 0.5 mg,  
1 ml); cDNA (5 ml) and distilled water (6 ml). Subsequently,  
5 additional distilled water (70 ml) and 10 mg/ml tRNA (0.1 ml) were  
added and the entire reaction was extracted through  
phenol:chloroform:isoamyl alcohol (25:24:1). The aqueous phase was  
removed, collected and diluted into 5M NaCl (10 ml) and absolute  
ethanol (-20°C, 250 ml). This was then centrifuged for 20 minutes  
at 14,000 x g, decanted, and the pellet resuspended into 70%  
10 ethanol (0.5 ml) and centrifuged again for 2 minutes at 14,000 x g.  
The DNA pellet was then dried in a speedvac and eluted into  
distilled water (3 ml) for use in the subsequent procedure.

The ligated cDNA/pRK5 vector DNA prepared previously was  
chilled on ice to which was added electrocompetent DH10B bacteria  
15 (Life Tech., 20 ml). The bacteria vector mixture was then  
electroporated as per the manufacturers recommendation.  
Subsequently SOC media (1 ml) was added and the mixture was  
incubated at 37°C for 30 minutes. The transformants were then  
plated onto 20 standard 150 mm LB plates containing ampicillin and  
incubated for 16 hours (37°C) to allow the colonies to grow.  
20 Positive colonies were then scraped off and the DNA isolated from  
the bacterial pellet using standard CsCl-gradient protocols.

An enriched 5'-cDNA library was then constructed to  
obtain a bias of cDNA fragments which preferentially represents the  
5' ends of cDNA's contained within the library. 10 mg of the  
pooled isolated full-length library plasmid DNA (41 ml) was  
combined with Not 1 restriction buffer (New England Biolabs, 5 ml)  
and Not 1 (New England Biolabs, 4 ml) and incubated at 37°C for one  
hour. The reaction was extracted through phenol:chloroform:isoamyl  
25 alcohol (25:24:1, 50 ml), the aqueous phase removed, collected and  
resuspended into 5M NaCl (5 ml) and absolute ethanol (-20°C, 150  
ml). This was then centrifuged for 20 minutes at 14,000 x g,  
decanted, resuspended into 70% ethanol (0.5 ml) and centrifuged  
again for 2 minutes at 14,000 x g. The supernatant was then  
30 removed, the pellet dried in a speedvac and resuspended in  
35

distilled water (10 ml).

The following reagents were brought together and incubated at 37°C for 2 hours: distilled water (3 ml); linearized DNA library (1 mg, 1 ml); Ribonucleotide mix (Invitrogen, 10 ml); transcription buffer (Invitrogen, 2 ml) and Sp6 enzyme mix. The reaction was then extracted through phenol:chloroform:isoamyl alcohol (25:24:1, 50 ml) and the aqueous phase was removed, collected and resuspended into 5M NaCl (5 ml) and absolute ethanol (-20°C, 150 ml) and centrifuged for 20 minutes at 14,000 x g. The pellet was then decanted and resuspended in 70% ethanol (0.5 ml), centrifuged again for 2 minutes at 14,000 x g, decanted, dried in a speedvac and resuspended into distilled water (10 ml).

The following reagents were added together and incubated at 16°C for 16 hours: 5X T4 ligase buffer (Life Tech., 3 ml); pRK5 Cla-Sal digested vector, 0.5 mg, 1 ml); cDNA (5 ml); distilled water (6 ml). Subsequently, additional distilled water (70 ml) and 10 mg/ml tRNA (0.1 ml) was added and the entire reaction was extracted through phenol:chloroform:isoamyl alcohol (25:24:1, 100 ml). The aqueous phase was removed, collected and diluted by 5M NaCl (10 ml) and absolute ethanol (-20°C, 250 ml) and centrifuged for 20 minutes at 14,000 x g. The DNA pellet was decanted, resuspended into 70% ethanol (0.5 ml) and centrifuged again for 2 minutes at 14,000 x g. The supernatant was removed and the residue pellet was dried in a speedvac and resuspended in distilled water (3ml). The ligated cDNA/pSST-amy.1 vector DNA was chilled on ice to which was added electrocompetent DH10B bacteria (Life Tech., 20 ml). The bacteria vector mixture was then electroporated as recommended by the manufacturer. Subsequently, SOC media (Life Tech., 1 ml) was added and the mixture was incubated at 37°C for 30 minutes. The transformants were then plated onto 20 standard 150 mm LB plates containing ampicillin and incubated for 16 hours (37°C). Positive colonies were scraped off the plates and the DNA was isolated from the bacterial pellet using standard protocols, e.g. CsCl-gradient.

The cDNA libraries were screened by hybridization with a

synthetic oligonucleotide probe:

GGGAGCCGCTCATGAGGAAGTTGGGCCTCATGGACAATGAGATAAAGGTGGCTAAAGCTGAGGCA  
GCGGG (SEQ ID NO:3) based on the EST.

Three cDNA clones were sequenced in entirety. The  
5 overlapping coding regions of the cDNAs were identical except for  
codon 410 (using the numbering system for Fig. 1); this position  
encoded a leucine residue (TTG) in both pancreatic cDNAs, and a  
methionine residue (ATG) in the kidney cDNA, possibly due to  
polymorphism.

10 The entire nucleotide sequence of Apo-2 is shown in  
Figure 1 (SEQ ID NO:2). Clone 27868 (also referred to as pRK5-Apo-  
2 deposited as ATCC \_\_\_\_\_, as indicated below) contains a single  
open reading frame with an apparent translational initiation site  
at nucleotide positions 140-142 [Kozak et al., supra] and ending at  
15 the stop codon found at nucleotide positions 1373-1375 (Fig. 1; SEQ  
ID NO:2). The predicted polypeptide precursor is 411 amino acids  
long, a type I transmembrane protein, and has a calculated  
molecular weight of approximately 45 kDa. Hydropathy analysis (not  
shown) suggested the presence of a signal sequence (residues 1-53),  
20 followed by an extracellular domain (residues 54-182), a  
transmembrane domain (residues 183-208), and an intracellular  
domain (residues 209-411) (Fig. 2A; SEQ ID NO:1). N-terminal amino  
acid sequence analysis of Apo-2-IgG expressed in 293 cells showed  
that the mature polypeptide starts at amino acid residue 54,  
25 indicating that the actual signal sequence comprises residues 1-53.

TNF receptor family proteins are typically characterized  
by the presence of multiple (usually four) cysteine-rich domains in  
their extracellular regions -- each cysteine-rich domain being  
approximately 45 amino acids long and containing approximately 6,  
30 regularly spaced, cysteine residues. Based on the crystal  
structure of the type 1 TNF receptor, the cysteines in each domain  
typically form three disulfide bonds in which usually cysteines 1  
and 2, 3 and 5, and 4 and 6 are paired together. Like DR4, Apo-2  
contains two extracellular cysteine-rich pseudorepeats (Fig. 2A),  
35 whereas other identified mammalian TNFR family members contain

three or more such domains [Smith et al., Cell, 76:959 (1994)].

The cytoplasmic region of Apo-2 contains a death domain (amino acid residues 324-391 shown in Fig. 1; see also Fig. 2A) which shows significantly more amino acid sequence identity to the death domain of DR4 (64%) than to the death domain of TNFR1 (30%); CD95 (19%); or Apo-3/DR3 (29%) (Fig. 2B). Four out of six death domain amino acids that are required for signaling by TNFR1 [Tartaglia et al., supra] are conserved in Apo-2 while the other two residues are semi-conserved (see Fig. 2B).

Based on an alignment analysis (using the ALIGN™ computer program) of the full-length sequence, Apo-2 shows more sequence identity to DR4 (55%) than to other apoptosis-linked receptors, such as TNFR1 (19%); CD95 (17%); or Apo-3 (also referred to as DR3, WSL-1 or TRAMP) (29%).

## EXAMPLE 2

### A. Expression of Apo-2 ECD

A soluble extracellular domain (ECD) fusion construct was prepared. An Apo-2 ECD (amino acid residues 1-184 shown in Figure 1) was obtained by PCR and fused to a C-terminal Flag epitope tag (Sigma). (The Apo-2 ECD construct included residues 183 and 184 shown in Figure 1 to provide flexibility at the junction, even though residues 183 and 184 are predicted to be in the transmembrane region). The Flag epitope-tagged molecule was then inserted into pRK5, and expressed by transient transfection into human 293 cells (ATCC CRL 1573).

After a 48 hour incubation, the cell supernatants were collected and either used directly for co-precipitation studies (see Example 3) or subjected to purification of the Apo-2 ECD-Flag by affinity chromatography on anti-Flag agarose beads, according to manufacturer's instructions (Sigma).

### B. Expression of Apo-2 ECD as an Immunoadhesin

A soluble Apo-2 ECD immunoadhesin construct was prepared. The Apo-2 ECD (amino acids 1-184 shown in Fig. 1) was fused to the

hinge and Fc region of human immunoglobulin G<sub>1</sub> heavy chain in pRK5 as described previously [Ashkenazi et al., Proc. Natl. Acad. Sci., 88:10535-10539 (1991)]. The immunoadhesin was expressed by transient transfection into human 293 cells and purified from cell supernatants by protein A affinity chromatography, as described by Ashkenazi et al., supra.

### EXAMPLE 3

#### Immunoprecipitation Assay Showing Binding Interaction Between Apo-2 and Apo-2 Ligand

To determine whether Apo-2 and Apo-2L interact or associate with each other, supernatants from mock-transfected 293 cells or from 293 cells transfected with Apo-2 ECD-Flag (described in Example 2 above) (5 ml) were incubated with 5 µg poly-histidine-tagged soluble Apo-2L [Pitti et al., supra] for 30 minutes at room temperature and then analyzed for complex formation by a co-precipitation assay.

The samples were subjected to immunoprecipitation using 25 µl anti-Flag conjugated agarose beads (Sigma) or Nickel-conjugated agarose beads (Qiagen). After a 1.5 hour incubation at 4° C, the beads were spun down and washed four times in phosphate buffered saline (PBS). By using anti-Flag agarose, the Apo-2L was precipitated through the Flag-tagged Apo-2 ECD; by using Nickel-agarose, the Apo-2 ECD was precipitated through the His-tagged Apo-2L. The precipitated proteins were released by boiling the beads for 5 minutes in SDS-PAGE buffer, resolved by electrophoresis on 12% polyacrylamide gels, and then detected by immunoblot with anti-Apo-2L or anti-Flag antibody (2 µg/ml) as described in Marsters et al., J. Biol. Chem., (1997).

The results, shown in Figure 3, indicate that the Apo-2 ECD and Apo-2L can associate with each other.

The binding interaction was further analyzed by purifying Apo-2 ECD from the transfected 293 cell supernatants with anti-Flag beads (see Example 2) and then analyzing the samples on a BIACORE™ instrument. The BIACORE™ analysis indicated a dissociation

constant ( $K_d$ ) of about 1 nM. BIACORE™ analysis also showed that the Apo-2 ECD is not capable of binding other apoptosis-inducing TNF family members, namely, TNF-alpha (Genentech, Inc., Pennica et al., Nature, 312:712 (1984), lymphotoxin-alpha (Genentech, Inc.), or Fas/Apo-1 ligand (Alexis Biochemicals). The data thus shows that Apo-2 is a specific receptor for Apo-2L.

#### EXAMPLE 4

##### Induction of Apoptosis by Apo-2

Because death domains can function as oligomerization interfaces, over-expression of receptors that contain death domains may lead to activation of signaling in the absence of ligand [Frazer et al., supra, Nagata et al., supra]. To determine whether Apo-2 was capable of inducing cell death, human 293 cells or HeLa cells (ATCC CCL 2.2) were transiently transfected by calcium phosphate precipitation (293 cells) or electroporation (HeLa cells) with a pRK5 vector or pRK5-based plasmids encoding Apo-2 and/or CrmA. When applicable, the total amount of plasmid DNA was adjusted by adding vector DNA. Apoptosis was assessed 24 hours after transfection by morphology (Fig. 4A); DNA fragmentation (Fig. 4B); or by FACS analysis of phosphatidylserine exposure (Fig. 4C) as described in Marsters et al., Curr. Biol., 6:1669 (1996). As shown in Figs. 4A and 4B, the Apo-2 transfected 293 cells underwent marked apoptosis.

For samples assayed by FACS, the HeLa cells were co-transfected with pRK5-CD4 as a marker for transfection and apoptosis was determined in CD4-expressing cells; FADD was co-transfected with the Apo-2 plasmid; the data are means  $\pm$  SEM of at least three experiments, as described in Marsters et al., Curr. Biol., 6:1669 (1996). The caspase inhibitors, DEVD-fmk (Enzyme Systems) or z-VAD-fmk (Research Biochemicals Intl.) were added at 200  $\mu$ M at the time of transfection. As shown in Fig. 4C, the caspase inhibitors CrmA, DEVD-fmk, and z-VAD-fmk blocked apoptosis induction by Apo-2, indicating the involvement of Ced-3-like proteases in this response.



FADD is an adaptor protein that mediates apoptosis activation by CD95, TNFR1, and Apo-3/DR3 [Nagata et al., supra], but does not appear necessary for apoptosis induction by Apo-2L [Marsters et al., supra] or by DR4 [Pan et al., supra]. A dominant-negative mutant form of FADD, which blocks apoptosis induction by CD95, TNFR1, or Apo-3/DR3 [Frazer et al., supra; Nagata et al., supra; Chinnayian et al., supra] did not inhibit apoptosis induction by Apo-2 when co-transfected into HeLa cells with Apo-2 (Fig. 4C). These results suggest that Apo-2 signals apoptosis independently of FADD. Consistent with this conclusion, a glutathione-S-transferase fusion protein containing the Apo-2 cytoplasmic region did not bind to *in vitro* transcribed and translated FADD (data not shown).

#### EXAMPLE 5

##### Inhibition of Apo-2L Activity by Soluble Apo-2 ECD

Soluble Apo-2L (0.5  $\mu$ g/ml, prepared as described in Pitti et al., supra) was pre-incubated for 1 hour at room temperature with PBS buffer or affinity-purified Apo-2 ECD (5  $\mu$ g/ml) together with anti-Flag antibody (Sigma) (1  $\mu$ g/ml) and added to HeLa cells. After a 5 hour incubation, the cells were analyzed for apoptosis by FACS (as above) (Fig. 4D).

Apo-2L induced marked apoptosis in HeLa cells, and the soluble Apo-2 ECD was capable of blocking Apo-2L action (Fig. 4D), confirming a specific interaction between Apo-2L and Apo-2. Similar results were obtained with the Apo-2 ECD immunoadhesin (Fig. 4D). Dose-response analysis showed half-maximal inhibition at approximately 0.3 nM Apo-2 immunoadhesin (Fig. 4E).

#### EXAMPLE 6

##### Activation of NF- $\kappa$ B by Apo-2

An assay was conducted to determine whether Apo-2 activates NF- $\kappa$ B.

HeLa cells were transfected with pRK5 expression plasmids encoding full-length native sequence Apo-2, DR4 or Apo-3 and

harvested 24 hours after transfection. Nuclear extracts were prepared and 1  $\mu$ g of nuclear protein was reacted with a  $^{32}$ P-labelled NF- $\kappa$ B-specific synthetic oligonucleotide probe

ATCAGGGACTTTCCGCTGGGGACTTTCCG (SEQ ID NO:4) [see, also, MacKay et al., *J. Immunol.*, 153:5274-5284 (1994)], alone or together with a 50-fold excess of unlabelled probe, or with an irrelevant  $^{32}$ P-labelled synthetic oligonucleotide

AGGATGGGAAGTGTGTGATATATCCTTGAT (SEQ ID NO:5). In some samples, antibody to p65/RelA subunits of NF- $\kappa$ B (1  $\mu$ g/ml; Santa Cruz Biotechnology) was added. DNA binding was analyzed by an electrophoretic mobility shift assay as described by Hsu et al., *supra*; Marsters et al., *supra*, and MacKay et al., *supra*.

The results are shown in Fig. 5. As shown in Fig. 5A, upon transfection into HeLa cells, both Apo-2 and DR4 induced significant NF- $\kappa$ B activation as measured by the electrophoretic mobility shift assay; the level of activation was comparable to activation observed for Apo-3/DR3. Antibody to the p65/RelA subunit of NF- $\kappa$ B inhibited the mobility of the NF- $\kappa$ B probe, implicating p65 in the response to all 3 receptors.

An assay was also conducted to determine if Apo-2L itself can regulate NF- $\kappa$ B activity. HeLa cells or MCF7 cells (human breast adenocarcinoma cell line, ATCC HTB 22) were treated with PBS buffer, soluble Apo-2L (Pitti et al., *supra*) or TNF-alpha (Genentech, Inc., see Pennica et al., *Nature*, 312:721 (1984)) (1  $\mu$ g/ml) and assayed for NF- $\kappa$ B activity as above. The results are shown in Fig. 5B. The Apo-2L induced a significant NF- $\kappa$ B activation in the treated HeLa cells but not in the treated MCF7 cells; the TNF-alpha induced a more pronounced activation in both cell lines. Several studies have disclosed that NF- $\kappa$ B activation by TNF can protect cells against TNF-induced apoptosis [Nagata, *supra*].

The effects of a NF- $\kappa$ B inhibitor, ALLN (N-acetyl-Leu-Leu-norleucinal) and a transcription inhibitor, cyclohexamide, were also tested. The HeLa cells (plated in 6-well dishes) were preincubated with PBS buffer, ALLN (Calbiochem) (40  $\mu$ g/ml) or

cyclohexamide (Sigma) (50  $\mu$ g/ml) for 1 hour before addition of Apo-2L (1  $\mu$ g/ml). After a 5 hour incubation, apoptosis was analyzed by FACS (see Fig. 5C).

The results are shown in Fig. 5C. Both ALLN and cyclohexamide increased the level of Apo-2L-induced apoptosis in the HeLa cells. The data indicates that Apo-2L can induce protective NF- $\kappa$ B-dependent genes. The data also indicates that Apo-2L is capable of activating NF- $\kappa$ B in certain cell lines and that both Apo-2 and DR4 may mediate that function.

#### EXAMPLE 7

##### Northern Blot Analysis

Expression of Apo-2 mRNA in human tissues was examined by Northern blot analysis. Human RNA blots were hybridized to a 4.6 kilobase  $^{32}$ P-labelled DNA probe based on the full length Apo-2 cDNA; the probe was generated by digesting the pRK5-Apo-2 plasmid with EcoRI. Human fetal RNA blot MTN (Clontech) and human adult RNA blot MTN-II (Clontech) were incubated with the DNA probes. Blots were incubated with the probes in hybridization buffer (5X SSPE; 2X Denhardt's solution; 100 mg/mL denatured sheared salmon sperm DNA; 50% formamide; 2% SDS) for 60 hours at 42°C. The blots were washed several times in 2X SSC; 0.05% SDS for 1 hour at room temperature, followed by a 30 minute wash in 0.1X SSC; 0.1% SDS at 50°C. The blots were developed after overnight exposure.

As shown in Fig. 6, a predominant mRNA transcript of approximately 4.6kb was detected in multiple tissues. Expression was relatively high in fetal and adult liver and lung, and in adult ovary and peripheral blood leukocytes (PBL), while no mRNA expression was detected in fetal and adult brain. Intermediate levels of expression were seen in adult colon, small intestine, testis, prostate, thymus, pancreas, kidney, skeletal muscle, placenta, and heart. Several adult tissues that express Apo-2, e.g., PBL, ovary, and spleen, have been shown previously to express DR4 [Pan et al., supra], however, the relative levels of expression of each receptor mRNA appear to be different.

#### EXAMPLE 8

##### Chromosomal Localization of the Apo-2 gene

Chromosomal localization of the human Apo-2 gene was examined by radiation hybrid (RH) panel analysis. RH mapping was performed by PCR using a human-mouse cell radiation hybrid panel (Research Genetics) and primers based on the coding region of the Apo-2 cDNA [Gelb et al., Hum. Genet., 98:141 (1996)]. Analysis of the PCR data using the Stanford Human Genome Center Database indicates that Apo-2 is linked to the marker D8S481, with an LOD of 11.05; D8S481 is linked in turn to D8S2055, which maps to human chromosome 8p21. A similar analysis of DR4 showed that DR4 is linked to the marker D8S2127 (with an LOD of 13.00), which maps also to human chromosome 8p21.

To Applicants' present knowledge, to date, no other member of the TNFR gene family has been located to chromosome 8.

\* \* \* \* \*

Deposit of Material

The following materials have been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, USA (ATCC):

<u>Material</u>	<u>ATCC Dep. No.</u>	<u>Deposit Date</u>
pRK5-Apo-2	_____	_____

This deposit was made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of a viable culture of the deposit for 30 years from the date of deposit. The deposit will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Genentech, Inc. and ATCC, which assures permanent and unrestricted availability of the progeny of the culture of the deposit to the public upon issuance of the pertinent U.S. patent or upon laying open to the public of any U.S. or foreign patent application, whichever comes first, and assures availability of the progeny to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC §122 and the Commissioner's rules pursuant thereto (including 37 CFR §1.14 with particular reference to 886 OG 638).

The assignee of the present application has agreed that if a culture of the materials on deposit should die or be lost or destroyed when cultivated under suitable conditions, the materials will be promptly replaced on notification with another of the same. Availability of the deposited material is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the

invention. The present invention is not to be limited in scope by the construct deposited, since the deposited embodiment is intended as a single illustration of certain aspects of the invention and any constructs that are functionally equivalent are within the scope of this invention. The deposit of material herein does not constitute an admission that the written description herein contained is inadequate to enable the practice of any aspect of the invention, including the best mode thereof, nor is it to be construed as limiting the scope of the claims to the specific illustrations that it represents. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

5 (i) APPLICANT: Ashkenazi, Avi J.

(ii) TITLE OF INVENTION: Apo-2 RECEPTOR

(iii) NUMBER OF SEQUENCES: 5

10

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Genentech, Inc.

(B) STREET: 460 Point San Bruno Blvd

(C) CITY: South San Francisco

(D) STATE: California

(E) COUNTRY: USA

(F) ZIP: 94080

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: 3.5 inch, 1.44 Mb floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: WinPatin (Genentech)

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE: 15-May-1997

(C) CLASSIFICATION:

30 (viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Marschang, Diane L.

(B) REGISTRATION NUMBER: 35,600

(C) REFERENCE/DOCKET NUMBER: P1101

35 (ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 415/225-5416

(B) TELEFAX: 415/952-9881

(C) TELEX: 910/371-7168

5 (2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 411 amino acids

(B) TYPE: Amino Acid

10 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Met Glu Gln Arg Gly Gln Asn Ala Pro Ala Ala Ser Gly Ala Arg  
1 5 10 15

Lys Arg His Gly Pro Gly Pro Arg Glu Ala Arg Gly Ala Arg Pro  
20 25 30

Gly Leu Arg Val Pro Lys Thr Leu Val Leu Val Val Ala Ala Val  
35 40 45

Leu Leu Leu Val Ser Ala Glu Ser Ala Leu Ile Thr Gln Gln Asp  
50 55 60

Leu Ala Pro Gln Gln Arg Ala Ala Pro Gln Gln Lys Arg Ser Ser  
65 70 75

Pro Ser Glu Gly Leu Cys Pro Pro Gly His His Ile Ser Glu Asp  
80 85 90

Gly Arg Asp Cys Ile Ser Cys Lys Tyr Gly Gln Asp Tyr Ser Thr  
95 100 105

His Trp Asn Asp Leu Leu Phe Cys Leu Arg Cys Thr Arg Cys Asp



60045615  
 20456159  
 251599  
 251599

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	Glu Met Cys Arg Lys Cys Arg Thr Gly Cys Pro Arg Gly Met Val		
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	Lys Val Gly Asp Cys Thr Pro Trp Ser Asp Ile Glu Cys Val His		
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	Leu Ile Val Ala Val Phe Val Cys Lys Ser Leu Leu Trp Lys Lys		
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	Pro Glu Arg Val Asp Arg Ser Ser Gln Arg Pro Gly Ala Glu Asp		
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	275	280	285

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Asn Glu Gly Asp Pro Thr Glu Thr Leu Arg Gln Cys Phe Asp Asp  
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Phe Ala Asp Leu Val Pro Phe Asp Ser Trp Glu Pro Leu Met Arg  
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10 Lys Leu Gly Leu Met Asp Asn Glu Ile Lys Val Ala Lys Ala Glu  
335 340 345

Ala Ala Gly His Arg Asp Thr Leu Tyr Thr Met Leu Ile Lys Trp  
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15 Val Asn Lys Thr Gly Arg Asp Ala Ser Val His Thr Leu Leu Asp  
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Asp His Leu Leu Ser Ser Gly Lys Phe Met Tyr Leu Glu Gly Asn  
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25 Ala Asp Ser Ala Xaa Ser  
410 411

(2) INFORMATION FOR SEQ ID NO:2:

- 30 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 1799 base pairs  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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CCACGGGCCT GAGAGACTAT AAGAGCGTTC CCTACCGCC ATG GAA 145

Met Glu

10

1

CAA CGG GGA CAG AAC GCC CCG GCC GCT TCG GGG GCC CGG 184

Gln Arg Gly Gln Asn Ala Pro Ala Ala Ser Gly Ala Arg

5

10

15

AAA AGG CAC GGC CCA GGA CCC AGG GAG GCG CGG GGA GCC 223

Lys Arg His Gly Pro Gly Pro Arg Glu Ala Arg Gly Ala

20

25

AGG CCT GGG CTC CGG GTC CCC AAG ACC CTT GTG CTC GTT 262

Arg Pro Gly Leu Arg Val Pro Lys Thr Leu Val Leu Val

30

35

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GTC GCC GCG GTC CTG CTG TTG GTC TCA GCT GAG TCT GCT 301

Val Ala Ala Val Leu Leu Leu Val Ser Ala Glu Ser Ala

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CTG ATC ACC CAA CAA GAC CTA GCT CCC CAG CAG AGA GCG 340

Leu Ile Thr Gln Gln Asp Leu Ala Pro Gln Gln Arg Ala

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55

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GCC CCA CAA CAA AAG AGG TCC AGC CCC TCA GAG GGA TTG 379

Ala Pro Gln Gln Lys Arg Ser Ser Pro Ser Glu Gly Leu

70

75

80

35



GTG GCT GTG TTT GTT TGC AAG TCT TTA CTG TGG AAG AAA 769  
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10 GGG GAC CCT GAG CGT GTG GAC AGA AGC TCA CAA CGA CCT 847  
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15 GGG GCT GAG GAC AAT GTC CTC AAT GAG ATC GTG AGT ATC 886  
 Gly Ala Glu Asp Asn Val Leu Asn Glu Ile Val Ser Ile  
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20 TTG CAG CCC ACC CAG GTC CCT GAG CAG GAA ATG GAA GTC 925  
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25 CAG GAG CCA GCA GAG CCA ACA GGT GTC AAC ATG TTG TCC 964  
 Gln Glu Pro Ala Glu Pro Thr Gly Val Asn Met Leu Ser  
 265 270 275

30 CCC GGG GAG TCA GAG CAT CTG CTG GAA CCG GCA GAA GCT 1003  
 Pro Gly Glu Ser Glu His Leu Leu Glu Pro Ala Glu Ala  
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35 GAA AGG TCT CAG AGG AGG AGG CTG CTG GTT CCA GCA AAT 1042  
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 290 295 300

GAA GGT GAT CCC ACT GAG ACT CTG AGA CAG TGC TTC GAT 1081  
 Glu Gly Asp Pro Thr Glu Thr Leu Arg Gln Cys Phe Asp  
 305 310

GAC TTT GCA GAC TTG GTG CCC TTT GAC TCC TGG GAG CCG 1120  
 Asp Phe Ala Asp Leu Val Pro Phe Asp Ser Trp Glu Pro  
 315 320 325

5 CTC ATG AGG AAG TTG GGC CTC ATG GAC AAT GAG ATA AAG 1159  
 Leu Met Arg Lys Leu Gly Leu Met Asp Asn Glu Ile Lys  
 330 335 340

10 GTG GCT AAA GCT GAG GCA GCG GGC CAC AGG GAC ACC TTG 1198  
 Val Ala Lys Ala Glu Ala Ala Gly His Arg Asp Thr Leu  
 345 350

TAC ACG ATG CTG ATA AAG TGG GTC AAC AAA ACC GGG CGA 1237  
 Tyr Thr Met Leu Ile Lys Trp Val Asn Lys Thr Gly Arg  
 355 360 365

GAT GCC TCT GTC CAC ACC CTG CTG GAT GCC TTG GAG ACG 1276  
 Asp Ala Ser Val His Thr Leu Leu Asp Ala Leu Glu Thr  
 370 375

CTG GGA GAG AGA CTT GCC AAG CAG AAG ATT GAG GAC CAC 1315  
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35 AGTAGGAAAG TGCCACAATT GTCACATGAC CGGTACTGGA AGAAACTCTC 1500

004515 DE 1500

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TTGGGCTACA TTGTAAGATC CATCTACAAA AAAAAAAAAA AAAAAAAAAAG 1750

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GGCGGCCGCG ACTCTAGAGT CGACCTGCAG AAGCTTGGCC GCCATGGCC 1799

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 70 base pairs
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGGAGCCGCT CATGAGGAAG TTGGGCCTCA TGGACAATGA GATAAAGGTG 50

GCTAAAGCTG AGGCAGCGGG 70

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ATCAGGGACT TTCCGCTGGG GACTTTCCG 29

5

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AGGATGGGAA GTGTGTGATA TATCCTTGAT 30

15



WHAT IS CLAIMED IS:

1. A method of modulating apoptosis in mammalian cells comprising exposing mammalian cells to Apo-2 polypeptide, said Apo-2 polypeptide having at least about 80% amino acid sequence identity with native sequence Apo-2 polypeptide comprising amino acid residues 1 to 411 of SEQ ID NO:1.

2. A method of modulating apoptosis in mammalian cells comprising exposing mammalian cells to an extracellular domain sequence of Apo-2 polypeptide comprising amino acid residues 54 to 182 of SEQ ID NO:1.

Abstract of the Disclosure

Novel polypeptides, designated Apo-2, which are capable of modulating apoptosis are provided. Compositions including Apo-2 chimeras, nucleic acid encoding Apo-2, and antibodies to Apo-2 are also provided.

5

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101	CCACGGGCCCT	GAGAGACTAT	AAGAGCGTTC	CCFACCGCCA	TGGACACAC	GGGACAGAAC	GCCCCGGCCG	CTTCGGGGGG	CCGAAAAAGG	CACGGGCCAG
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55	LeuileThr	GlnGlnAspL	euAlaProG1	nGlnArgAla	AlaProGlnG	lnLysArgSe	rSerProSer	GluclyLeuC	ysProProG1	yHisHisile
401	TCAGAAAGACG	GTAGAGATTG	CATCTCCTGC	AAATATGGAC	AGGACTATAG	CACTCACTGG	AATGACCTCC	TTTTCTGCTT	GGCTGCACC	AGGTGTGATT
	AGTCTTCTGC	CATCTCTAAC	GTAGAGGACG	TTTATACCTG	TCCTGATATC	GTGAGTGACC	TTACTGGAGG	AAAAGACGAA	CGCGACGTGG	TCCACACTAA
88	SerGluAspG	lyArgAspCy	sileSerCys	LysTyrglyG	lnAspTyrs	rThrHisTrp	AsnAspLeuL	eupheCysLe	uArgCysThr	ArgCysAspSer
501	CAGGTGAAGT	GGAGCTAAGT	CCCTGCACCA	CGACACAGAA	CACAGTGTGT	CAGTCCGAAG	AAGGCACCTT	CCGGGAAGAA	GATTCTCCTG	AGATGTGCCG
	GTCCACTTCA	CCTCGATTCA	GGGACGTGGT	GCTGGTCTTT	GTGTACACA	GTACACGCTC	TTCCGTGGAA	GGCCCTTCTT	CTFAGAGGAC	TCTACACGGC
122	GlyGluva	IgluLeuser	ProCysThrt	hrThrArgas	nThrValcys	GlnCysGluG	luGlyThrPh	eArgGluGlu	AspSerProG	luMetCysArg
601	GAAGTCCCGC	ACAGGGTGTC	CCAGAGGGAT	GGTCAAGGTC	GGTGAATTGA	CACCCTGGAG	TGACATCGAA	TGTGTCCACA	AAGAATCAGG	CATCATCATATA
	CTTCACGGCG	TGTCCACACG	GGTCTCCCTA	CCAGTTCCAG	CCACTAAACAT	GTGGGACCTC	ACTGTAGCTT	ACACAGGTGT	TTCTTAGTCC	GTAGTAGTAT
155	LysCysArg	ThrGlyCysP	roArgGlyMe	tVallyVal	GlyAspCyst	hrProTrpse	rAspIleGlu	CysValHisL	ysGluSerG1	yIleIleIle
701	GGAGTCAACG	TTGCAGCCGT	AGTCTTGTAT	GTGGCTGTGT	TTGTTTGCAA	GTCTTTACTG	TGGAAGAAAAG	TCCTTCCTTA	CCTGAAAGGC	ATCTGCTCAG
	CCTCAGTGTG	AACGTGGGCA	TCAGAACTAA	CACCGACACA	AACAAAAGTT	CAGAAAATGAC	ACCTTCTTTC	AGGAAGGAAT	GGACTTTCCG	TAGACGAGTC
188	GlyValThrV	alAlaAlava	lValLeuille	ValAlaValP	heValCysly	sserLeuLeu	TrpLysLysV	alleuProTy	rLeuLysGly	ileCysSerGly
801	GTGGTGGTGG	GGACCCCTGAG	CGTGTGGACA	GAAGCTCACA	ACGACCTGGG	GCTGAGGACA	ATGTCCTCAA	TGAGATCGTG	AGTATCTTGC	AGCCCCACCCA
	CACCAACACC	CCTGGGACTC	GCACACCTGT	CTTCGAGTGT	TGCTGGACCC	CGACTCCTGT	TACAGGAGTT	ACTCTAGCAC	TCATFAGAACG	TCGGGTGGGT
222	GlyGlyG1	yAspProGlu	ArgValaspA	rgSerSerG1	nArgProGly	AlaGluaspA	snValleuAs	nGluIleVal	serIleLeuG	lnProThrGln
901	GGTCCCTGAG	CAGGAAATGG	AAGTCCAGGA	GCCAGCAGAG	CCAACAGGTG	TCAACATGTT	GTCCCCCGGG	GAGTCAGAGC	ATCTGCTGGA	ACCGGCAGAA
	CCAGGACTC	GTCCTTTTACC	TTCAGGTCTC	CGGTGCTCTC	GGTTGTCAC	AGTTGTACAA	CAGGGGGCCC	CTCAGTCTCG	TAGACGACCT	TGGCCGCTCTT
255	ValProGlu	GlnGluMetG	luValGlnG1	uProAlaGlu	ProThrGlyV	alAsnMetLe	userProGly	GluserGluH	isLeuLeuG1	uProAlaGlu
1001	GCTGAAAAGT	CTCAGAGGAG	GAGGCTGCTG	GTTCCAGCAA	ATGAAGGTGA	TCCCCTGAG	ACTCTGAGAC	AGTCTTCCGA	TGACTTTTGA	GACTTGGTGC
	CGACTTTCCA	GAGTCTCCTC	CTCCGACGAC	CAAGGTGCGT	TACTTCCACT	AGGCTGACTC	TGAGACTCTG	TCACGAAGCT	ACTGAAACGT	CTGAACCCAG
288	AlaGluArgS	erglnNArgar	gArgLeuLeu	ValProAlaA	snGluGlyAs	pProThrGlu	ThrLeuArgg	lnCysPheAs	paspPheAla	AspLeuValPro

1101 CCTTTGACTC CTGGGAGCCG CTGATGAGGA AGTTGGGCCT CATGGACAAT GGGCTAAAGC TGAGGCAGCG GGCCACAGGG ACACCTTGTA  
 GGAACCTGAG GACCTCGGC GAGTACTCCT TCAACCCGGA GTACCTGTTA CTCTATTTCG ACTCCGTGCG CCGGTGTCCT TGTGGAACAT  
 322 PheAspse rTrpGluPro LeuMetArgL ysLeuGlyLe uMetAspAsn GluileLysV alAlaLysAl aGluAlaAla GlyHisArga spThrLeuTyr  
 1201 CACGATGCTG NTAAAGTGGG TCAACAAAAC CGGGCGAGAT GCCTCTGTCC ACACCCCTGCT GGATGCCCTTG GAGACGCTGG GAGAGAGACT TGCCAAAGCAG  
 GTGCTACGAC TATTTCACCC AGTTGTTTGT GCCCGCTCTA CGGAGACAGG TGTGGGACGA CCTACGGAAC CTCTGCGACC CTCTCTCTGA ACGGTTGCTC  
 355 ThrMetLeu ileLysTrpV alAsnLysTh rGlyArgAsp AlasrValH isthrLeuLe uAspAlaLeu GluThrLeuG lyGluArgLe uAlaLysGln  
 1301 AAGATTGAGG ACCACTTGTT GAGCTCTGGA AAGTTCATGT ATCTAGAAGG TAATGCAGAC TCTGCCWGTG CCTAAGTGTG ATTCTCTTCA GGAAGTGAGA  
 TTCTAACTCC TGGTGAACAA CTCGAGACCT TTCAAGTACA TAGATCTTCC ATTACGTCTG AGACGGAACA GGATTCACAC TAAGAGAAAGT CCTTCACTCT  
 388 LysileGlua spHisLeuLe uSerSerGly LysPheMetT yrLeuGluGl yAsnAlaAsp SerAlaXqQs erOC\*  
 1401 CCTTCCCTGG TTTACCTTTT TTCTGGAAAA AGCCCAACTG GACTCCAGTC AGTAGGAAAG TGCCACAATT GTCACATGAC CGGTACTGGA AGAAACTCTC  
 GGAAGGGACC AAATGGAAAA AAGACCTTTT TCGGGTTGAC CTGAGGTCAG TCATCCTTTC ACGGTGTTAA CAGTGTACTG GCCATGACCT TCTTTGAGAG  
 1501 CCATCCAACA TCACCCAGTG GATGGAACAT CCTGTAACTT TTCACTGCAC TTGGCATTTAT TTTTATAAGC TGAATGTGAT AATAAGGACA CTATGGAAAT  
 GGTAGGTTGT AGTGGGTCAC CTACCTTGTA GGACATTGAA AAGTGACGTG AACCGTAATA AAAATATTTCG ACTTACACTA TTATTCTCTGT GATACCTTTA  
 1601 GTCTGGATCA TTCCGTTTGT GCGTACTTTG AGATTGTTT TGGGATGTCA TTGTTTTTTC AGCAGTTTTT TATCCTAATG TAAATGCTTT ATTATTTTAT  
 CAGACCTAGT AAGGCAACA CGCATGAAAC TCTAAACCAA ACCCTACAGT AACAAAAAGTG TCGTGAAAAA ATAGGATTAC ATTTACGAAA TAAATAAATA  
 1701 TTGGGCTACA TTGTAAGATC CATCTACAA AAAAAA AAAA AAAA AAAA AAAA AAAA AAAA AAAA AAAA AAAA AAAA AAAA AAAA AAAA AAAA AAAA  
 AACCCGATGT AACATCTAG GTAGATGTTT TTTTTTTTTT TTTTTTTTTT CCGCCGGCGC TGAGATCTCA GCTGGACGTC TTCGAACCGG CGGTACCGG

Fig. 1 (cont.)

Fig. 2 A

1 MEQRGONAPAAAGARKRHGPGPREARGARGLRVPKTLVLVAAVLLLVSAESALITQQD  
61 LAPQORAAPOQKRSSPSEGLCPPGHHISEDGRDCISCKYQDYSTHWNDLLFCLLRCTRQD  
121 SGEVELSPCTTTRNTVCQCEEGTFREEDSPEMCRKRTGQPRGMVKGDCPTWSDIECVH  
181 KESGIIIGVTVAAVLIVAVFCKSLMKVLPYLKICSGGGGDPERVDRSSQRPGAED  
241 NVLNEIVSILQPTQVPEQEMEVEQPAETGVNMLSPGESEHLLLEPAEAERSQRRLLVPA  
301 NEGDPTEITLRQCFFDDFADLVFDFSWEPMLRKLGLMDNEIKVAKAEAAAGHRDITLYTMLIKW  
361 VNKTGRDASVHTIILDALETLGERIAKQKIEDHLLSSGKFMYLEGNADSALS

Fig. 2 B

Apo2	FADLVPPFDSWEP	*LMRKLGIM	*DNEIKVAKAEAA	--GHRD	TL
DR4	FANIVPPFDSW	QDLMRQLDI	TKNETDVVRAGTA	--GPGD	AL
Apo3/DR3	VMDAVPARRWK	EPVRTLGI	REAEIEAVEVEIGR	--FRDQ	Q
TNFR1	VVENVPPLRWK	EPVRTLGI	SDHFI	DRLELQNGR	-CLREAQ
Fas/Apo1	IAGVMTLSQV	KGVKKNGV	NEAKIDEIKNDN	VQDTAEQKV	

Apo2	VTMLIKKVVNKTGRD	-ASVHTLI	DALETLGERLAKQKIED
DR4	YAMIMKVVNKTGRN	-ASVHTLI	DALETLGERLAKQKIED
Apo3/DR3	YEMIKRRRRQQQP	--AGLGAVYA	ATERRMGLDGCVEEDLRS
TNFR1	YSMLATNRRRT	PPREATTIEL	IGRVLRDMDLLGLCEDEE
Fas/Apo1	-QILLRNWHQLH	GKKKAY-D	TIKKKANTCTLAENKTQT

Fig. 3

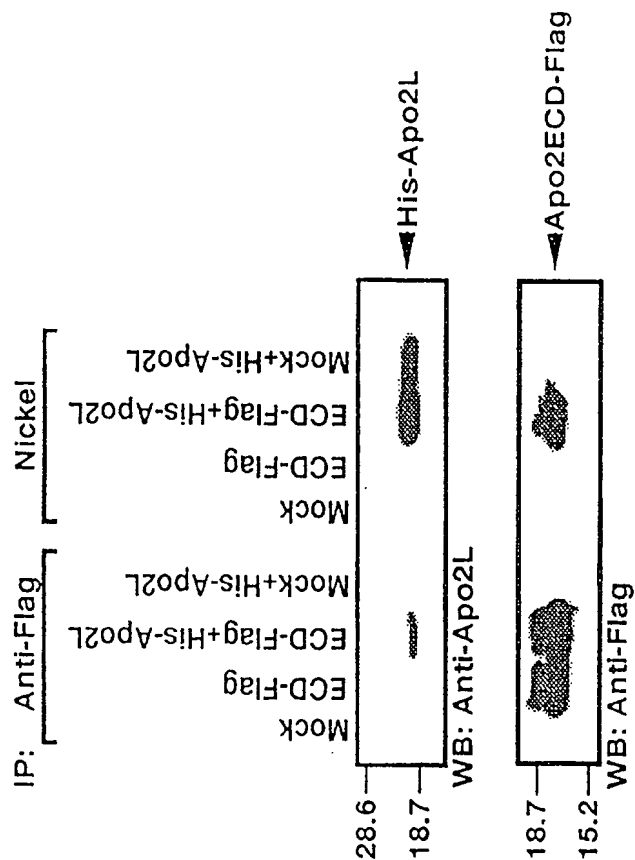
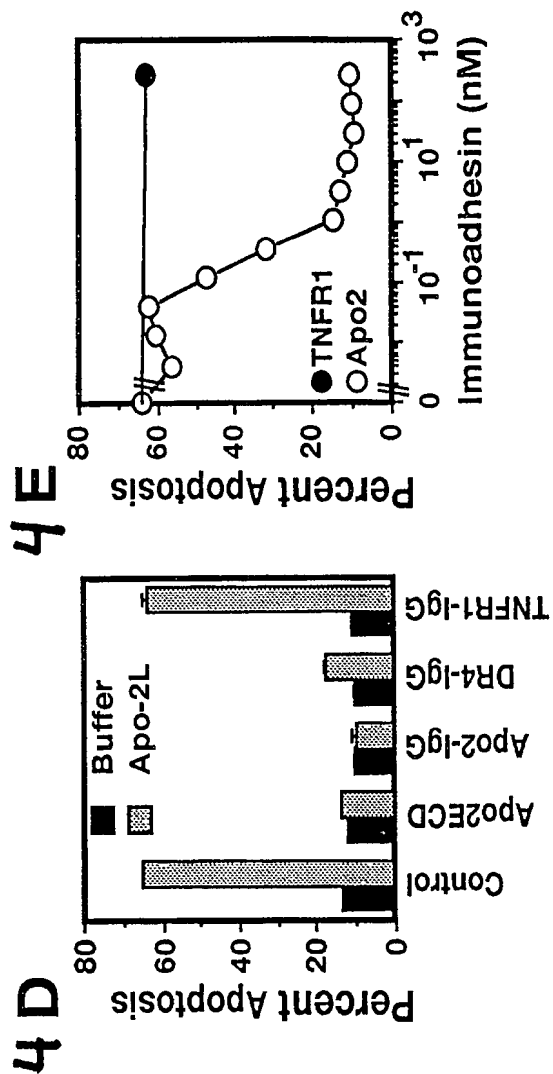
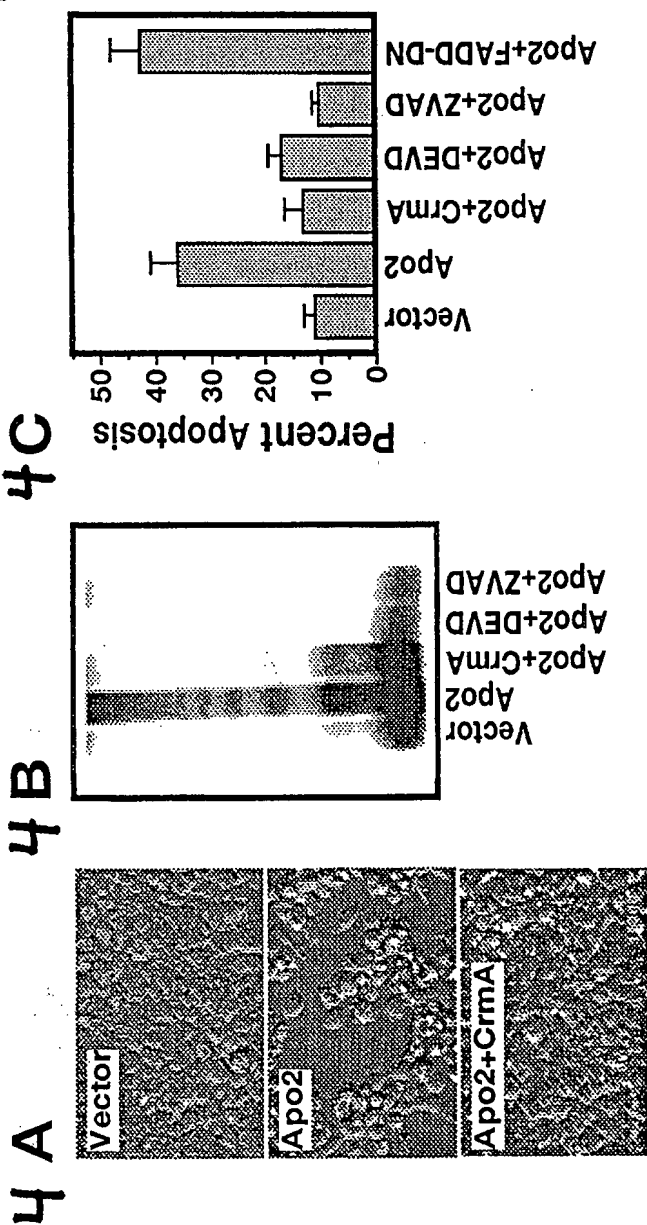


Fig. 4



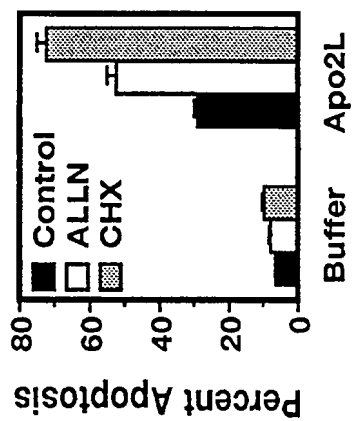
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Fig. 5



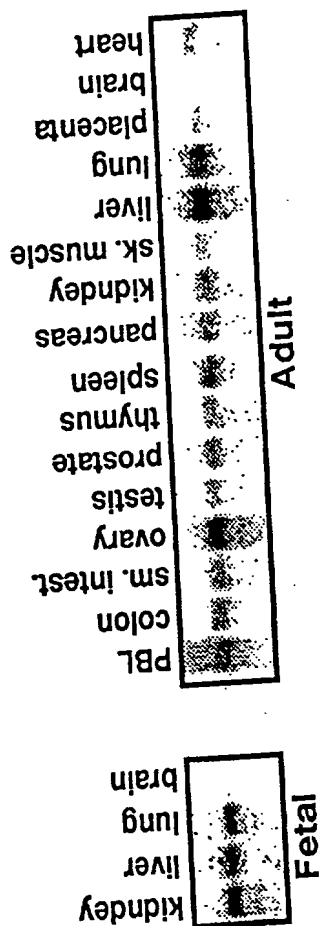


FIG. 6

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From Page No. 59

From Page No.

WV#

**Sub-4**

So far then

1. 1353959,

## 2. Cluster

### 3. Cluster

[illegible][illegible]

I asked Scot Marsters to design probes/primers in order to ~~clone~~ <sup>clone</sup> out the full-length cDNA. This appears clearly to be a death-domain containing protein.

Tissues: Metastatic Lung cancer (apparently from thyroid) LUNG.TM72  
Pancreatic islet cells ISL.TM01

To Page No.

Witnessed & Understood by me

Date \_\_\_\_\_

Invented by Am. Chenier

Date \_\_\_\_\_

Recorded by An. Advaitan

Witnessed & Under

TITLE

Project No.  
Book No. 22265

61

From Page No. \_\_\_\_\_

wlv, AM, incyte sequences

1

To: wlv

From: andgene.com (Avi Ashkenazi)

Subject: incyte sequences

So far there are 3 sets of Incyte sequences that we plan to clone out:

1. 1353959, 1353903, (cluster 78530), homologous to Apo-2L
2. Cluster 16411 (10 sequences), homologous to Apo-2L
3. Cluster 75799 (2 sequences), homologous to the Apo-3 death domain

To Page No. \_\_\_\_\_

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Book No. 22265

TITLE ~~Anti~~ Death Domain protein

TITLE

~~Anti~~

From Page No. \_\_\_\_\_

From Page No. \_\_\_\_\_

Positive phage clones have been identified in two  
libraries - pancreas and \_\_\_\_\_, using a probe based  
on the death-domain-like signal sequence. They  
One clone of each library was sent to sequencing.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by

Ari Ashkenazi

Recorded by

Ari Ashkenazi

Date \_\_\_\_\_

Witnessed &amp; Understood by me, \_\_\_\_\_



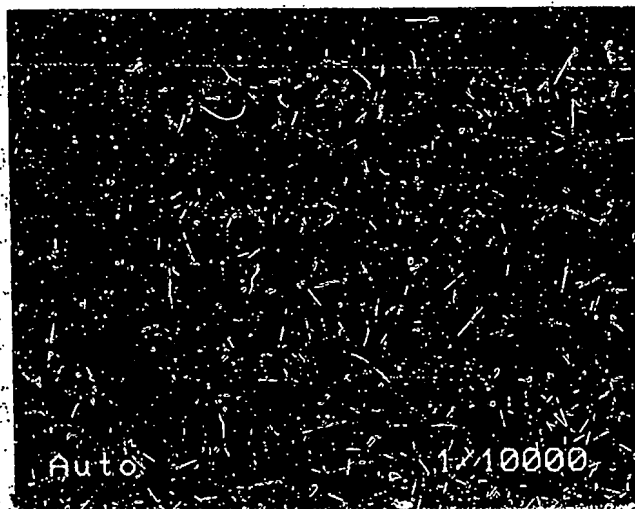
TITLE Death Domain protein

Project No. 22265  
Book No. 22265

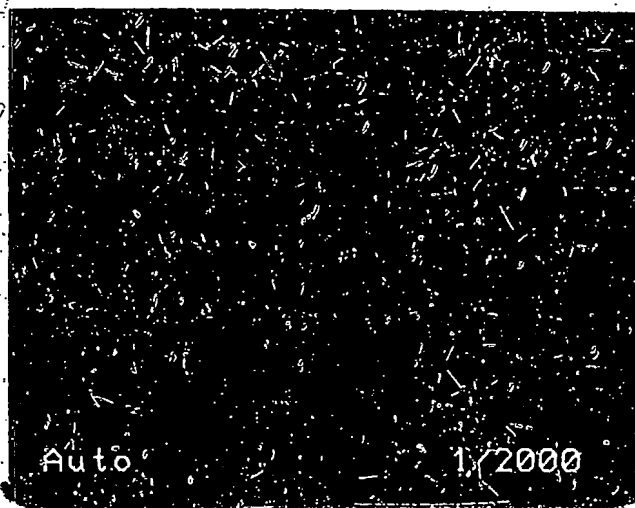
63

From Page No.     

Today, we obtained evidence for activation of apoptosis by the  
new death domain protein. So, transfected 293 cells with  
control PRK5 of phase clone subcloned into PRK5



Control  
PRK5  
transfected  
cells  
@ 24h



Death Domain  
cDNA-  
PRK5  
transfected  
cells  
@ 24h

To Page No.     

Witnessed & Understood by me,     

Date     

Invented by     

Date     

Recorded by Am. Ishikawa

Recorded by \_\_\_\_\_



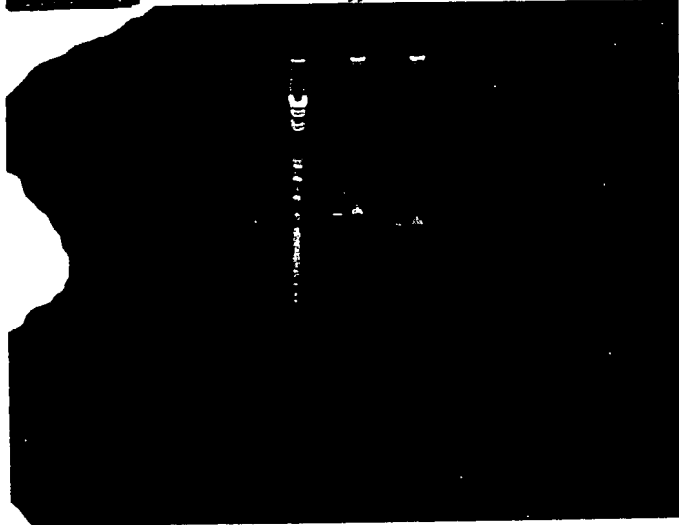


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ISSUED TO Robert Pitti  
ON [REDACTED] 19 [REDACTED]  
DEPARTMENT Mol. Oncology  
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fusion PCR productsgel formatmumLioSioLSBST 5 cycles DD2CFLio Sio L S

To Page No. \_\_\_\_\_

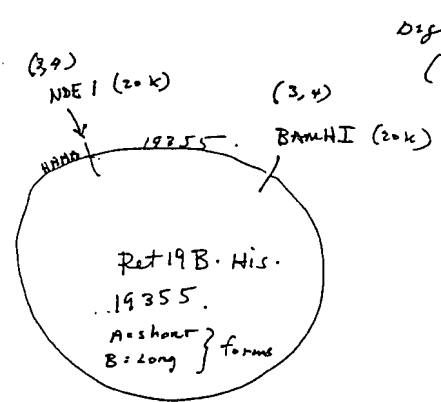
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Date

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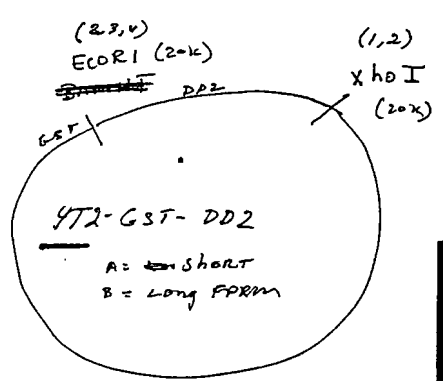
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# Cell format for Plasmid digest



Digest Buffer ~~+~~  
(BamHI Buffer)

X 10ml 4T2	X 3 Ret19B.
X 2ml R1	X 2ml NDE I
X 2ml xho I	X 2ml BAMHI
X 12ml Buffer R1	12ml Bam Buffer
X 76ml dH <sub>2</sub> O	83ml dH <sub>2</sub> O



Digest Buffer ~~+~~  
(EcoRI Buffer)

Ret19B



## Cell format

1. MW
2. —
3. Ret19B
4. Ret19B
5. —
6. 4T2
7. 4T2

Ret19B was cut out of agarose and stored at 4°C.

Witnessed & Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by [Signature]

Date \_\_\_\_\_

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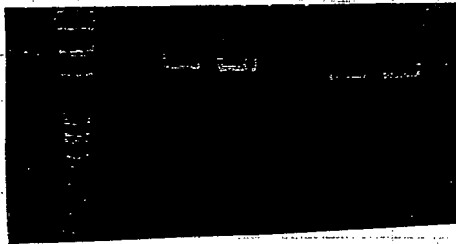
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To Page No. \_\_\_\_\_

From Page No. \_\_\_\_\_

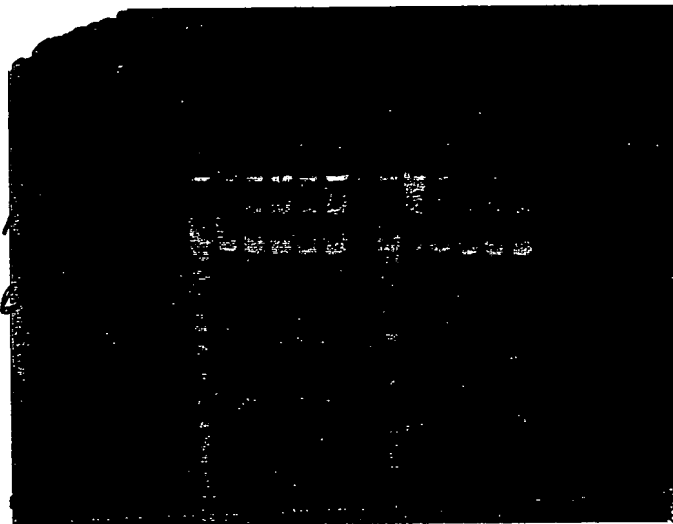
6% Acrylamide + then electro-eluted

10 L 510 S



The 10 L and 510 S were cloned into pGEM6  
(The L+S were as well, but these were disregarded)

The pGEM clones were PCR selected & then EcoRI/XhoI  
digested, 5 of each. Of these 4 going and  
3 don't were submitted for sequence.



To Page No. \_\_\_\_\_

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3/17/97

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{ A. L<sub>10</sub> } long 10 + 15 cycle PCR  
 { B. L<sub>15</sub> }  
 { C. S<sub>10</sub> } short 10 + 15 cycle PCR  
 { D. S<sub>15</sub> }  
 short + low recovery

TH Buffer 1  $\mu$ L ✓  
 VECTOR 1  $\mu$ L ✓  
 PCR 5  $\mu$ L ✓  
 Ligase 1  $\mu$ L ✓  
 dH<sub>2</sub>O 2  $\mu$ L ✓  
 10  $\mu$ L TOTAL

3 hours @ 15°C.

2, 3, 4, 5, 6

4.

50, 51, 52, 53, 54

To Page No. \_\_\_\_\_

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3/18/97

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6 cm plates of confluent 293's were TFX'd  
in Calcium Phosphate procedure with the  
following Dna:

plate	Receptor	Conc	PRKF
1	10 DD2	0	
2	10 TNFR1	0	
3	10 APD3	0	
4	10 DD2	10	
5	10 TNFR1	10	
6	10 APD3	10	
7	10 PRK5	0	
8	10 PRK5	10	

3 Plates were TFX'd for each and incubated o/n  
at 37°C.

Lysis

1. Cells were spun and washed with PBS
2. Cells were lysed with

1% NP40 10mM TRIS

150mM NaCl

PMSE, Aprotinin etc.

for 15 minutes.

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To Page No. \_\_\_\_\_

Date

3/19/92

From Page No. \_\_\_\_\_

1:50 dilutions of sample were assayed at 720mg/ml.  
or 1mg/ml final (These samples exceeded drc  
by at least 2 fold. See Below for BCA  
analysis

STD BSA (um)

1	1000mg/ml	OD 562 nm
2	500	(1.388) .780
3	250	.451
4	125	.244
5	62.5	.140
6	31.25	.080
7	15.625	.046
8	Q	Q

Sample  
OD 562 (1:20)

1	(327) .518	$\times 20 = 6.54 \text{ mg/ml}$
2	(195) .314	$\times 20 = 3.90 \text{ mg/ml}$
3	(324) .513	$\times 20 = 6.48 \text{ mg/ml}$
4	(344) .544	$\times 20 = 6.88 \text{ mg/ml}$
5	(377) (294) .594	$\times 20 = 7.54 \text{ mg/ml}$
6	(294) .467	$\times 20 = 5.88 \text{ mg/ml}$
7	(302) .479	$\times 20 = 6.04 \text{ mg/ml}$
8	(258) .412	$\times 20 = 5.2 \text{ mg/ml}$

$$y = .0319 + .00154x$$

$$\frac{y - .0319}{.00154} = x$$

6.54 mg/mL

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

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Date \_\_\_\_\_

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3/19/97



TITLE PCR confirmation

Project No. \_\_\_\_\_

Book No. 26610

75

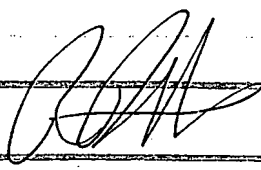
From Page No. \_\_\_\_\_

~~2.5~~  
5ul P1 P1  
5ul P2 P2  
25ul TAQ  
5ul Buffer  
12ul dNTP  
✓ 30.00ul dH<sub>2</sub>O  
50ul TOTAL

To Page No. \_\_\_\_\_

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Date \_\_\_\_\_

Invented by 

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

3/19/07

From Page No. \_\_\_\_\_

1% high melt agarose

4T2 P6EX vector 300 µg/mL

10 µL Vexton

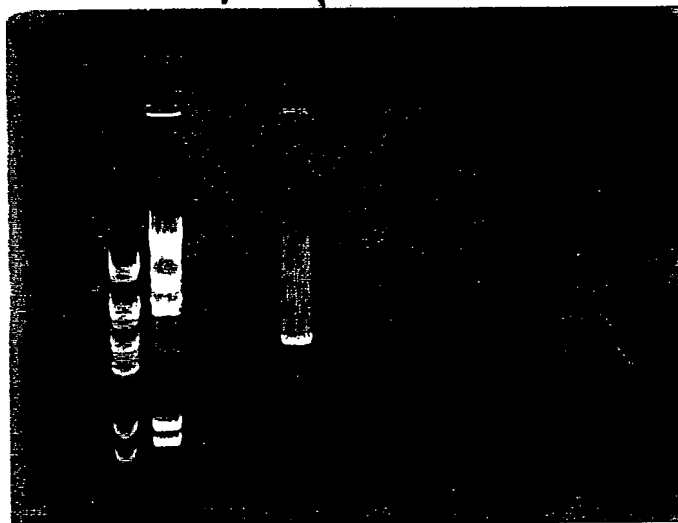
10 µL ECOR1 Buffer

3 µL ECOR1

3 µL Xho1

74 dH<sub>2</sub>O

100 µL



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Date \_\_\_\_\_

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Recorded by \_\_\_\_\_

To Page No. \_\_\_\_\_

Date

3/24/97

RI - xho1 cuts  
TITLE EST- DP2 L&S PHEM

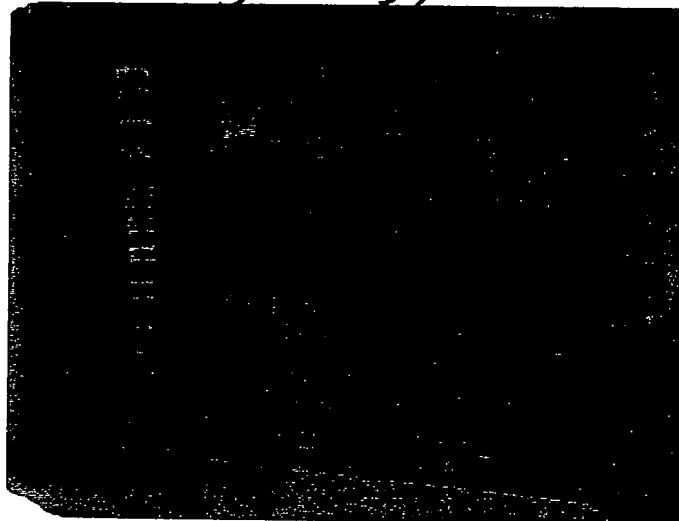
Project No. \_\_\_\_\_  
Book No 26610

77

From Page No. \_\_\_\_\_

Purpose to EC21 / xho cut PHEM  
clones  
selected & p/c clean'd.

L. 5 S. 54



10ul PHEM  
5ul Bsp  
5ul L&S  
5ul xho  
29ul H<sub>2</sub>O  
50ul

dd2long	1	VPRG	SPGIPANEGDPTETLRQCFDDFADLVPFDSWEPLMRKLGMDNEIK
DD25.54.m13f	1	-----L	ADLVPFDSWEPLMRKLGMDNEIK
DD2L.5.m13f.rc	1	-----	SPGIPANEGDPTETLRQCFDDFADLVPFDSWEPLMRKLGMDNEIK
dd2long	51	VAKAEAAGHRDTLYTMLIKWVNKTGRDASVHTLLDALETGGERLAKQKIE	
DD25.54.m13f	26	VAKAEAAGHRDTLYTMLIKWVNKTGRDASVHTLLDALETGGERLAKQKIE	
DD2L.5.m13f.rc	47	VAKAEAAGHRDTLYTMLIKWVNKTGRDASVHTLLDALETGGERLAKQKIE	
dd2long	101	DHLLSSGKFMYLEGNADSALS	-----
DD25.54.m13f	76	DHLLSSGKFMYLEGNADSALSXVLERPNHXCGRLOVDHMGELPTRWMHSL	
DD2L.5.m13f.rc	97	DHLLSSGKFMYLEGNADSALSXVLERPQS	-----

P1 files made from sequencing lab files -  
dd2 long is PHEM DNA sequence.

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Date

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To Page No. \_\_\_\_\_

3/24/97

From Page No. \_\_\_\_\_

Procedure

1. after EtoH ppt of each DNA, bring up each in 19  $\mu$ L of Buffer 2
2. molar ratio of Vector to insert is  $\frac{1}{3}$

3. add to the vial

1  $\mu$ L of vector } both diluted in vial 2 buffer  
 5  $\mu$ L of PCR prod }  
 4  $\mu$ L of ~~PCR~~ dilution Buffer (vial 2)  
 19  $\mu$ L of lig. Buffer (2x) vial 1

mix thoroughly

add 1  $\mu$ L of ligase (vial 3)

mix thoroughly

inc. at 5' at RT.

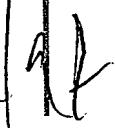
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Date \_\_\_\_\_

Invented by  \_\_\_\_\_

Recorded by \_\_\_\_\_

Date

3/26/97 

To Page No. \_\_\_\_\_

From Page No. \_\_\_\_\_

10-80

These were done from Acyl. Purified  
Phen - PCR frago. & agarose purified  
plasmid 4T2. followed by  
P/c cleanup

Witnessed &amp; Understood by me,

Date

Invented by

Date

Recorded by

To Page No. \_\_\_\_\_

3/31/92

TITLE 4T2-p6ex DD2 clones  
6P2-p6ex DD2 clones

Project No. \_\_\_\_\_  
Book No 26610

83

From Page No. \_\_\_\_\_

I Purpose: to subclone EcoRI — DD2 — xhoI  
fragment from p6ex into 6P2-p6ex  
vector.

## II. materials:

A. Clone #5 Parent. Long version of GST-DD2  
see P. 70

B. Clone #54 Parent. Short version of GST-DD2  
see P. 70

See P. 77 of this book for sequence  
confirmation of fragments.

## III. procedure

4T2-p6ex AN

1. 6P2-p6ex vector was cut with EcoRI/xhoI  
as follows:

100  $\mu$ L vector (6P2 + 4T2)

100  $\mu$ L R1 Buffer

3  $\mu$ L R1

3  $\mu$ L xhoI

74  $\mu$ L dH<sub>2</sub>O

100  $\mu$ L

to cut for 2 hours at 37°C and then Supplied for 2 hrs  
Run on 1% agarose large gel at 100 V.  
and cleaned up with

SAP (sodium alkaline phosphatase)

and cleaned up with Quick purification  
System.

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4/3/92

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2. Phem- $\gamma$  claus were ( $\times 5 \times 54$ ) were cut as above in vector section and run out on 2% mini-agarose gels at 100V + cleaned up with guaraged Extaend

3. Ligation was done o/n with T4 ligase at  $16^{\circ}\text{C}$ . (NEB 202CL)

1 mL vector  
5 mL insert  
1 mL T4 ligase  
5 mL Ligase Buffer  
38 mL of  $\text{H}_2\text{O}$   
59 mL TOTAL

4.) Transformed into DH10B high competent cells.

5 mL of ligation  
66 mL of cells  
30' @  $0^{\circ}\text{C}$  (ice)  
45" @  $42^{\circ}\text{C}$   
2' @  $0^{\circ}\text{C}$  (ice)  
500  $\mu\text{L}$  of RT SOC

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4+2-p6EX DD2 cloni  
6P2-p6EX DD2 cloni

Project No. \_\_\_\_\_  
Book No. 26610

85

TITLE

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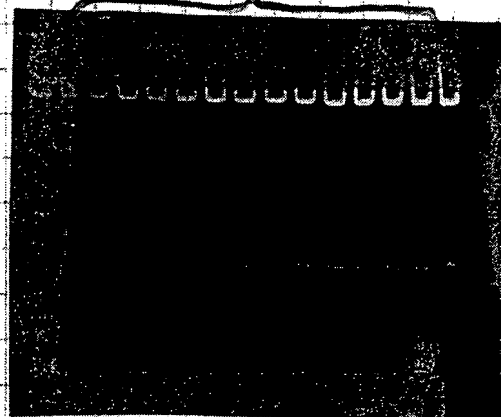
4. conet

1 hour at 37°C on shaker  
plated out on LB carb plate O/N at 37°C.

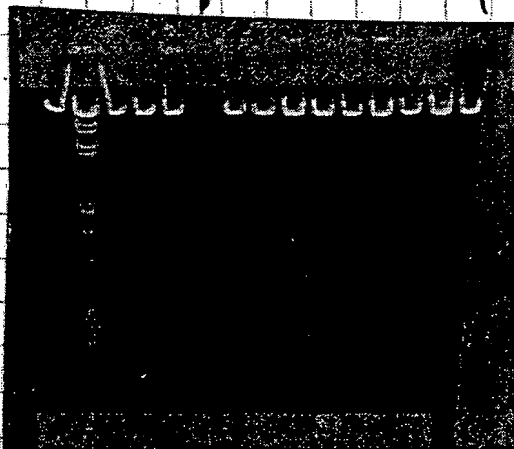
5. Rigor confirmation on 18 clones of each  
6P2 construct (Long+short)

120 µL D20A  
100 µL ELOX 1  
100 µL XhoI  
25 µL RT Buffer  
10.5 µL dH<sub>2</sub>O

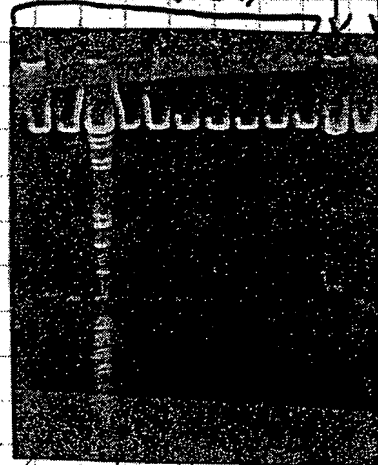
6P2-Long-p6EX



6P2-Long 6P2-Short



6P2-Short  
p6EX #6  
XhoI #1



Conclusion: Subcloning  
was successful.  
All clones positive -

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Date \_\_\_\_\_



From Page No. \_\_\_\_\_

Expression: 0.659 when IPTG was addedA<sub>600</sub> →

@ 12:45 pm

9:15 - 12:45 pm

**Expression of GST fusions in E.Coli****Materials:**

Ice Cold PBS

2YT/Carb

IPTG (200 mM) Genentech Stock. This 200x off the shelf.

20% Triton X 100. Prepare from 100% stock at least 1 hour ahead

**Procedure****A. Growth and Extraction**

1. Inoculate 100 mL of 2YT with a colony of E.coli cells containing pGEX plasmid and incubate overnight at 37 degrees C.
2. Warm 2YT/carb media to 37 degrees C and dilute the stock E.Coli 1:100 and grow to A<sub>600</sub> of .5-2.
3. Add IPTG 1:2000 and incubate an additional 2-6 hours
4. Transfer to 500 ml bottles and centrifuge for 10 minutes at 7,700 g. (GS-3 at 7000 rpm) at 4 degrees C.
5. Drain the pellet and place on ice immediately.
6. Resuspend the pellet in ice cold PBS, 50 mL per Liter of original culture volume. Transfer to 50 mL conicals.
7. Adjust volume of suspension to about 40 ml per tube. Sonicate on ice twice for 25 seconds on #5 using the large probe. Save an aliquot of the sonicate for GST CDNB assay at this point. Assay bacteria from pre-sonicate along side sonicate to see that the protein has been released.
8. Add 20 % Triton X-100 to a final concentration of 1% (2.6 mL per 50 mL of sonicate). Mix gently for 30 minutes at 4 degrees C.
9. Transfer to Oakridge tubes and spin at 12,000 g for 10 minutes at 4 degrees C.
10. Transfer the supernatant to 50 ml conicals. This can be frozen at this step.

**B. Purification**

1. Preparation of 50 % slurry ( will need: 1 ml of GST-Sepharose 50% slurry for each Liter of original culture volume).

A. Gently shake the bottle of gel to resuspend.

B. Pipette 1.33 ml of slurry for each liter of original culture volume.

C. Sediment the gel by centrifuging at 500 g for 5 minutes.

D. Wash each 1.33 mL of slurry with 10 mL of cold PBS and repeat centrifugation.

E. For each 1.33 mL of slurry resuspend with 1 mL of PBS to yield a final concentration of 50% slurry.

2. Add slurry to the supernatant (1 mL of slurry for each liter of original culture volume) and incubate with gentle agitation at RT for 30 minutes.
3. Centrifuge at 500 X for 5 minutes to sediment the gel. Remove the supernatant and save for assay.
4. Carefully load gel onto a column. Do not trap air bubbles
5. Gravity wash 3x 10 mL with PBS per ml of resin. Check flow-through with Bradford (Note BCA and Lowry cannot be done here after because of the presence of glutathione).
6. Elute with 1 mL of Glutathione Elution Buffer per 2 ml of 50% slurry. Add directly to the column and mix with resin. Let stand at RT for 10 minutes then drip through into clean eppendorf tubes.
7. Repeat elution at least twice. Peak elution may not be the first one, so these repeated elutions may be critical. Place fractions on ice immediately and assay be CDNB and Bradford.

See Page 89 - 90  
for commercial  
procedure

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4/10/97

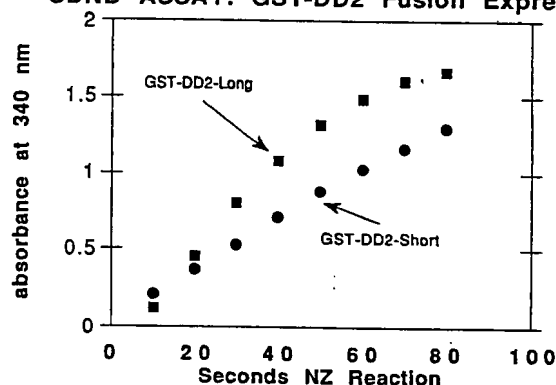
From Page No. \_\_\_\_\_

CDNB assay

GST DD2 - Short Long

10	.209	.328
20	.368	.659
30	.525	1.010
40	.708	1.290
50	.880	1.531
60	1.029	1.698
70	1.164	1.815
80	1.30	1.875
90	1.445	1.946
100	1.536	1.972
110	1.643	2.003
120	1.746	2.033
130	1.827	2.030
140	1.872	—
150	1.958	—
160	1.994	—
170	2.055	—
180	2.077	—

CDNB ASSAY: GST-DD2 Fusion Expressor



See page 89  
for protocol

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BSA mg/mL		Abs 595	value	
0		0		
15.		037		
31.25		059		
62.5		102	$y = m_0 + m_1 * x$	
125		186		
250		316	$m_0 = .0292$	
500		532	$m_1 = .00104529$	
1000		844		
				mg/mL
S <sub>1</sub>	1:10	.090	$y = .0292$	58.2
S <sub>2</sub>	1:10	.171	$.001045$	136.0
S <sub>3</sub>	1:10	.140	75 mL elutions	106
S <sub>4</sub>	1:10	.085		53.4
			$\Sigma$	
L <sub>1</sub>	1:10	.218	75 mL elutions	180.7
L <sub>2</sub>	1:10	.258		218.9
L <sub>3</sub>	1:10	.189		153
L <sub>4</sub>	1:10	.087		55.3
			$\Sigma$	
Total yield		total		
S <sub>1</sub>	.582	x.75	.4365	
S <sub>2</sub>	1.360	"	1.020	$\Sigma = 2.65$
S <sub>3</sub>	1.060	"	.795	
S <sub>4</sub>	.534	"	.4005	
		$\Sigma$	$= 2.65$	
L <sub>1</sub>	1.807	"	1.35	$\Sigma = 4.56 \text{ mg}$
L <sub>2</sub>	2.189	"	1.642	
L <sub>3</sub>	1.530	"	1.15	
L <sub>4</sub>	.553	"	.415	
		$\Sigma$	$=$	

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CDNB assay

From Page No. 87

**Procedure A: Measurement of GST Activity by CDNB Assay**

Before beginning this procedure, you will need to prepare Glutathione solution, as follows: Add 5 ml of sterile water to the bottle containing Reduced Glutathione powder. Mix until the powder dissolves. Aliquot into microcentrifuge tubes and store at -20°C until needed. Avoid more than five freeze-thaw cycles.

- Note: Samples appropriate for the CDNB assay include bacterial sonicates, column eluates, etc. It is critical that pGEX-bearing cells be lysed prior to performing the CDNB assay.

**Standard Assay**

- In a microcentrifuge tube, combine the following:  

Distilled water	880 µl
10X Reaction Buffer	100 µl
CDNB	10 µl
Glutathione solution*	10 µl
<b>Total Volume</b>	<b>1000 µl</b>
- Cap and mix by inverting the tube several times.
- Note: Due to its low solubility in water, CDNB may cause the solution to become slightly cloudy. The solution should clear upon mixing.
- Transfer 500 µl volumes of the above solution into two UV-transparent cuvettes. To one of these cuvettes ("sample cuvette") add the sample to be assayed (5-50 µl). To the other cuvette ("blank cuvette"),

\* Prepared as described at the start of this procedure.

8

add a volume of 1X Reaction Buffer equal to the amount of sample added to the sample cuvette.

- Cover each cuvette with wax film and invert to mix.
- Place the blank cuvette in a UV spectrophotometer and blank at 340 nm. Measure the absorbance of the sample cuvette at 340 nm and simultaneously start a stopwatch or other timer.
- Record absorbance readings at 340 nm at one-minute intervals for 5 minutes by first blanking the spectrophotometer with the blank cuvette and then measuring the absorbance of the sample cuvette.
- Note: The reaction rate is linear provided an  $A_{340}$  of approximately 0.8 is not exceeded during the five-minute time course. Initially, results may be plotted to verify that the reaction rate is linear over the time course. Adjust the amount of sample containing the GST fusion protein to maintain a linear reaction rate.
- Note: Under the conditions of the assay, glutathione and CDNB will react spontaneously to a small degree, in the absence of GST, to form a chemical moiety that absorbs at 340 nm. Under standard assay conditions and volumes at 22°C, the baseline drift produced by this reaction causes a  $\Delta A_{340}/\text{min}$  of approximately 0.003 (or 0.015 in 5 minutes). This reaction is corrected for by blanking the spectrophotometer with the blank cuvette before each reading of the sample cuvette.

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**PROTOCOL****Introduction**

The following protocol can be conveniently scaled to purify as little as 80 µg or as much as 400 mg of GST fusion protein using Glutathione Sepharose 4B (27-4574-01).

Yield of fusion protein is highly variable and is affected by the nature of the fusion protein, the host cell, and the culture conditions used. Fusion protein yields can range from 1-3 mg/l up to 10 mg/l (2). Table 1 can be used to approximate culture volumes based on an average yield of 2.5 mg/l.

- Note: The reagent volume requirements are based on a binding capacity of 8 mg of recombinant GST per ml of drained gel, which is the minimum binding capacity of Glutathione Sepharose 4B (27-4574-01). Refer to the label on the bottle for the exact binding capacity.

Table 1: Reagent volume requirements for different protein yields.

Component	80 mg	16 mg	1.6 mg	80 µg
Culture Volume	20 liters	4 liters	400 ml	20 ml
Volume Sonicate	1000 ml	200 ml	20 ml	1 ml
Glutathione Sepharose				
Bed Volume*	10 ml	2 ml	200 µl	10 µl
1X PBS**	100 ml	20 ml	2 ml	100 µl
Glutathione				
Elution Buffer	10 ml	2 ml	200 µl	10 µl

\*To obtain the desired bed volume, use twice the volume of 50% Glutathione Sepharose slurry prepared in Procedure A (i.e. 1 ml of 50% Glutathione Sepharose slurry will give a bed volume of 0.5 ml).

\*\*This volume is "per wash." Three washes are required.

4 8mg

**Procedure A: Preparation of 50% Slurry of Glutathione Sepharose 4B**

Glutathione Sepharose 4B may be used for batch or column purification of glutathione S-transferases or recombinant GST fusion proteins produced using the pGEX series of expression vectors. Once the gel is equilibrated, it can be transferred to a suitable chromatography column, if desired (Empty Disposable Columns PD-10, Pharmacia code number 17-0435-01, have a total volume capacity (includes gel and sample) of ~13 ml). See Procedure C for protocols for column purification of Glutathione S-transferase proteins.

- Referring to Table 1 on page 4, determine the bed volume of Glutathione Sepharose 4B required for your application. Glutathione Sepharose 4B as supplied is approximately a 75% slurry. The following procedure results in a 50% slurry.
- Gently shake the bottle of Glutathione Sepharose 4B to resuspend the gel.
- Use a pipet to remove sufficient slurry for use and transfer to an appropriate container/tube. Dispense 1.33 ml of the original Glutathione Sepharose 4B slurry per ml of bed volume required. One ml of drained gel is capable of binding at least 8 mg of recombinant GST. Refer to the label on the bottle for the exact binding capacity.
- Sediment the gel by centrifugation at 500 x g for 5 minutes. Carefully decant the supernatant.
- Wash the Glutathione Sepharose 4B by the addition of 10 ml of cold (4°C) 1X PBS (see Additional Reagents and Materials, Appendix 1) per 1.33 ml of the original slurry of Glutathione Sepharose 4B dispensed. Invert to mix.

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Batch load

- Note: Glutathione Sepharose 4B must be thoroughly washed with 1X PBS to remove the 20% ethanol storage solution. Residual ethanol may interfere with subsequent procedures.
- Sediment the gel by centrifugation at 500 x g for 5 minutes. Decant the supernatant.
- For each 1.33 ml of the original slurry of Glutathione Sepharose 4B dispensed, add 1 ml of 1X PBS. This results in a 50% slurry. Mix well prior to subsequent pipetting steps.
- Note: Glutathione Sepharose 4B equilibrated with 1X PBS may be stored at 4°C for up to 1 month.

This  
part was  
done after  
loading onto a  
column.

### Procedure B: Batch Purification of Glutathione S-Transferase Proteins

#### Binding

- Add 2 ml of the 50% slurry of Glutathione Sepharose 4B equilibrated with 1X PBS (Procedure A) to each 100 ml of bacterial sonicate (Appendix 4).
- Incubate with gentle agitation at room temperature for 30 minutes.
  - Note: At this stage, the gel with adsorbed fusion protein may be packed into a Disposable Column to facilitate washing and elution steps. If a Disposable Column is used, refer to Procedure C for instructions on washing and elution.
- Centrifuge the suspension at 500 x g for 5 minutes to sediment the gel. Remove the supernatant.
  - Note: The majority of the eluate can be discarded. However, a sample should be retained for analysis by SDS-PAGE or CDNB assay (see GST Detection Module, 27-4590-01) to measure the efficiency of binding to the gel.
- Wash the Glutathione Sepharose 4B peller with 10 bed volumes\* of 1X PBS.
- Centrifuge the suspension at 500 x g for 5 minutes to sediment the gel. Discard the wash.
- Repeat the wash twice for a total of three washes.
  - Note: Protein bound to the gel may be eluted directly at this stage using Glutathione Elution Buffer (see Appendix 1). If desired, GST fusion proteins may be cleaved while still bound to the gel with Thrombin or Factor Xa to liberate the protein of interest from the GST moiety. If the pGEX construct contains the recognition sequence for Thrombin refer to Appendix 5: Thrombin Cleavage of GST Fusion Proteins.

\*Bed volume is equal to 0.5 X the volume of the 50% Glutathione Sepharose slurry used.

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Column Wash & Elution

- Note: Glutathione Sepharose 4B must be thoroughly washed with 1X PBS to remove the 20% ethanol storage solution. Residual ethanol may interfere with subsequent procedures.
- Note: Glutathione Sepharose 4B equilibrated with 1X PBS may be stored at 4°C for up to 1 month.

#### Binding

- Use a pipet to apply the bacterial sonicate (Appendix 4) to the gel in a drained and equilibrated Glutathione Sepharose 4B column.
  - Note: If needed, the sonicate may be clarified by filtration through a 0.45 µm filter before applying it to the column.
- Remove the end cap or Parafilm and allow the sonicate to flow through.
  - Note: The majority of the eluate can be discarded. However, a sample should be retained for analysis by SDS-PAGE or CDNB assay (see GST Detection Module, 27-4590-01) to measure the efficiency of binding to the gel.
- Wash the gel by the addition of 10 bed volumes\* of 1X PBS. Allow the column to drain. Repeat twice more for a total of three washes.
  - Note: Protein bound to the gel may be eluted directly at this stage using Glutathione Elution Buffer (see Appendix 1). If desired, GST fusion proteins may be cleaved while still bound to the gel with Thrombin or Factor Xa to liberate the protein of interest from the GST moiety. If the pGEX construct contains the recognition sequence for Thrombin refer to Appendix 5: Thrombin Cleavage of GST Fusion Proteins.

\*Bed volume is equal to 0.5 X the volume of the 50% Glutathione Sepharose slurry used.

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#### Elution

- Once the column with bound protein has been washed and drained, replace the bottom cap or wrap with Parafilm.
- Elute the fusion protein by the addition of 1 ml of Glutathione Elution Buffer (prepared as described in Appendix 1) per ml bed volume\*. Incubate the column at room temperature (22-25°C) for 10 minutes to elute the fusion protein.
- Remove the bottom cap or Parafilm and collect the eluate. This contains the fusion protein.
- Repeat the elution and collection steps twice more. Pool the three eluates.
  - Note: Following the elution steps, a significant amount of fusion protein may remain bound to the gel. Volumes and times used for elution may vary among fusion proteins. Additional elutions may be required. Eluates should be monitored for GST fusion protein by SDS-PAGE or by CDNB assay (GST Detection Module, 27-4590-01).
  - Note: The yield of fusion protein can be estimated by measuring the absorbance at 280 nm. For the GST affinity tag, 1 A<sub>280</sub> = 0.5 mg/ml.
  - Note: The yield of protein may also be determined by standard chromogenic methods (e.g. Lowry, BCA, Bradford, etc.). If a Lowry or BCA type method is to be used, the sample must first be dialyzed against 2000 volumes of 1X PBS to remove glutathione, which can interfere with protein measurement. The Bradford method can be performed in the presence of glutathione.

\*Bed volume is equal to 0.5 X the volume of the 50% Glutathione Sepharose slurry used.

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Date \_\_\_\_\_

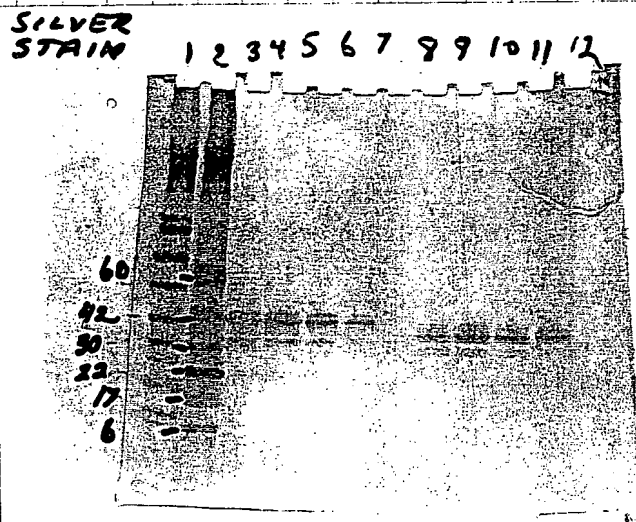
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Date 11/1/97

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4-20% GRADIENT 26610-91

lane

- |    |                        |                   |
|----|------------------------|-------------------|
| 1  | NOVEX SEE BLUE MARKERS |                   |
| 2  | NOVEX MULTIMARK        |                   |
| 3  | 21                     | } Running at 40kd |
| 4  | 42                     |                   |
| 5  | 23                     |                   |
| 6  | 24                     |                   |
| 7  | —                      |                   |
| 8  | 51                     | } Running at 35kd |
| 9  | 52                     |                   |
| 10 | 53                     |                   |
| 11 | 54                     |                   |
| 12 | —                      |                   |

GST alone runs at

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4/15/96

From Page No. —

A 260

Pharmic

based on  
2000 bp  
saturation

1	.139	(21)	1.39 mg/ml	0.92 mg/ml
2	.200	(38)	2.0 mg/ml	1.32 mg/ml
3	.206	(56)	2.04 mg/ml	1.36
4	.120	(81)	1.20 mg/ml	.792

all of these were Ethanol ppt & re-solubilized  
in dH<sub>2</sub>O to 1 µg/7.5 µl or

$$\frac{1.33 \mu\text{g}}{\text{mL}}$$

Total mass

Total

Brought up in ...  
dH<sub>2</sub>O

* 1	.045 mL x	.920 $\frac{\text{mg}}{\text{mL}}$	=	41.5 µg	312 µL
* 2	.045 mL x	1.32 $\frac{\text{mg}}{\text{mL}}$	=	59.4 µg	447 µL
* 3	.045 mL x	1.36 $\frac{\text{mg}}{\text{mL}}$	=	61.2 µg	460 µL
* 4	.045 mL x	.792 $\frac{\text{mg}}{\text{mL}}$	=	35.6 µg	268 µL

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Date

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14  
Page No

**NOTEBOOK NO.** 27510  
**ISSUED TO** Robert Pitti  
**ON** 4-18 **19** 97.  
**DEPARTMENT** Molecular Oncology  
**RETURNED** 19

—SCIENTIFIC NOTEBOOK CO.—  
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616-429-8285

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TITLE IN VITRO TRANSLATION of

1

From Page No. \_\_\_\_\_

Purpose is to look at in vitro translation of AP02-EC0-Flagg.  
Constructs 26610-96.

(21) 38, 56, 81 were all diluted to 133  $\mu$ g/ml and used in the reticulocyte lysate <sup>TNT</sup> assay.

The entire translate was ppt'd by adding 39  $\mu$ l of BSA blocked Kodak 2-FLAGG Resin.

[M2 AFFINITY Gel Cat 1B13020]

for 1 hour at 40°C. This was then washed extensively in lysis buffer. (also used for binding) and then eluted with 49  $\mu$ l of Hot Sample buffer for 4 minutes @ 100°C. Entire load was put on a

4-20% gradient and SDS-PAGE.

Exposure at RT is for 20 minutes on Fuji Screen.

all lanes translated  
\* 38 looks less, but is artifact of lower load due to lysis spill.

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Invented by

Date

Recorded by

4/18/96

From Page No. \_\_\_\_\_

I. Purpose: to precipitate apo2L band to in vitro translation expressed material.

@ 10ug/ml.

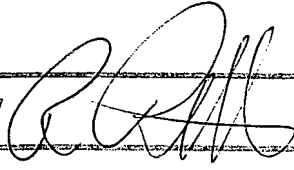
		PPT
1. + apo	DD2 ECD	N <sub>2</sub> <sup>24</sup>
2. -	DD2 ECD	"
3. +	DR4 - ECD	"
4. -	DR4 - ECD	"
5. +	CDC 42 Flag	"
6. -	CDC 42 Flag	"
7. +	CTRL UT	"
8. -	CTRL UT	"
<del>9. -</del>	<del>CTRL UT</del>	<del>"</del>

9. +	DD2 ECD	α Flag.
10. +	UT	α Flag.

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Date \_\_\_\_\_

Invented by 

Date

Recorded by \_\_\_\_\_

7/21/92

From Page No. \_\_\_\_\_

Purpose: to purify Flag-DDR2 for binding experiments -

Procedure

1. 3 mL of commercial m2 suspension was loaded onto a column & drained to top of resin.
2. Bed was washed at 4 mL/min in TBS for approx 10 mins.
3. Resin was then re-suspended by pipetting with 3 x 5 mL of glycine pH 3.5 and allowed to settle to interface.
4. Column was then washed at 2 mL/min with TBS until pH of 7.4 was achieved & sustained.
5. ~150 mL of filtered supernatant was loaded at 2 mL/min.
6. Resin was washed to baseline with TBS.
7. Elution with Glycine 0.1 M pH 3.5 at a flow-rate of 0.5 mL/min. 1 minute fractions were collected & assayed by A280 & Cuvette in SDS 4-20% gradient.

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Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

1/24/97

TITLE Purification of FLAG-DD2

Project No. \_\_\_\_\_  
Book No. 27510

5

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0806/N50/24726/9.90

A<sub>280</sub>

Fraction pool 7+8 .037  
" 9+10 .081  
" 11+12 .025

100 nM

$\frac{100 \text{ nmol/L}}{\text{LITER}} = \frac{22.999 \text{ ng}}{\text{nmole}}$   
22.999 ng/mL

.2 meter  
.1 V Rev.

1 mL/min carb sp. 5 mL/min

FLAG ECD DD2

1M Glycine  
PH 3.5

+ 8.0 PH TRIS

Boil

.5 mL fractions

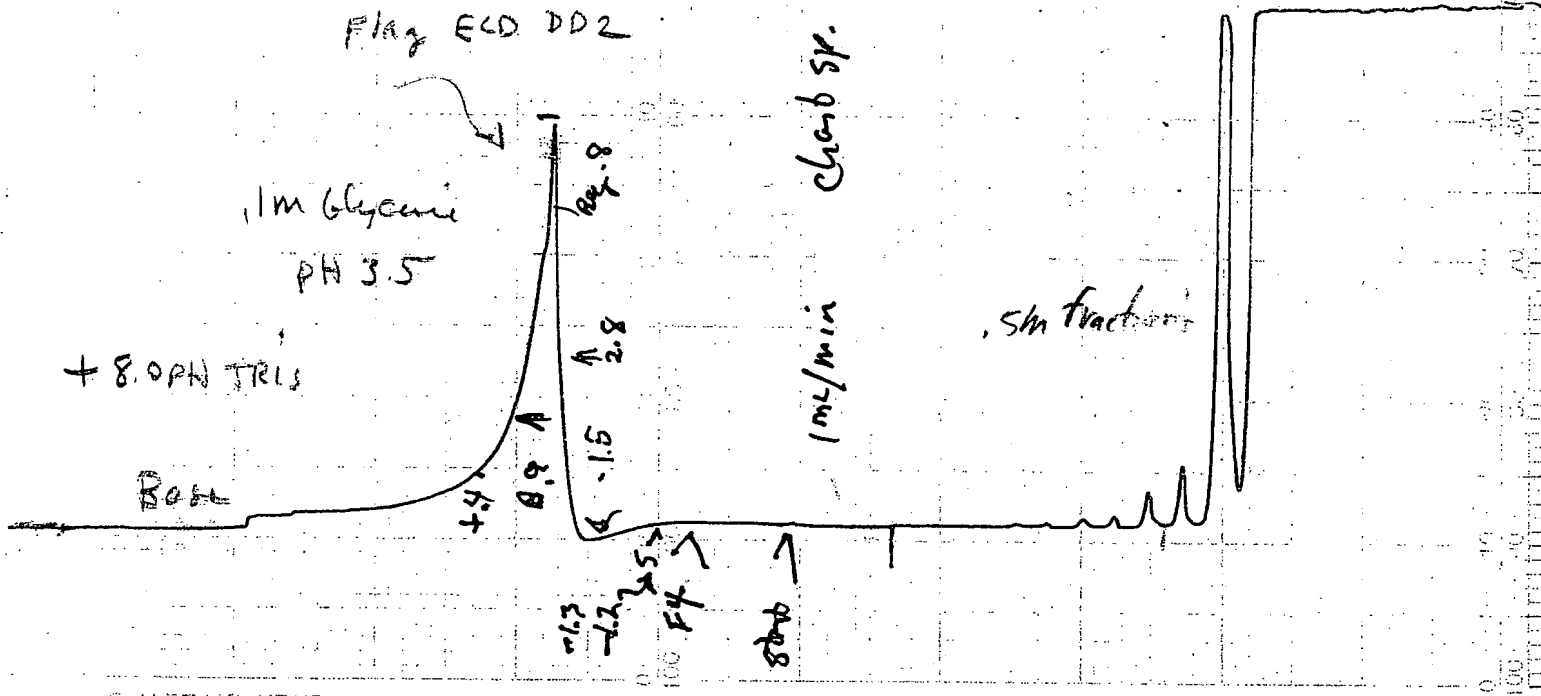


CHART NO. K7XP-9

Witnessed & Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Recorded by \_\_\_\_\_

Date \_\_\_\_\_

To Page No. \_\_\_\_\_

*[Handwritten signature]*  
4/24/98

From Page No. \_\_\_\_\_

Purpose: to prepare purified IgG-AP02

materials

A 1 L of of transfected supernatant from  
Seco Master 4/28/92B. Bpligen, 21 mg/mL capacity 150 cm/hr load  
rate (2.5 cm/minute)

Procedure:

1. a 1.0 cm (dia) column was loaded with 3.0 mL bed volume and pooled @ 2.0 mL/min with TBS after column was allowed to gravity drain to head.
2. Column was cycled with TBS <sup>and</sup> 50 mM citrate pH 3.0 and sample was loaded at 1-2 mL/minute
3. Column was washed to baseline with TBS and eluted with 50 mM citrate pH 3.0.
4. column was cycled with 6M guanidine and TBS to regenerate.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

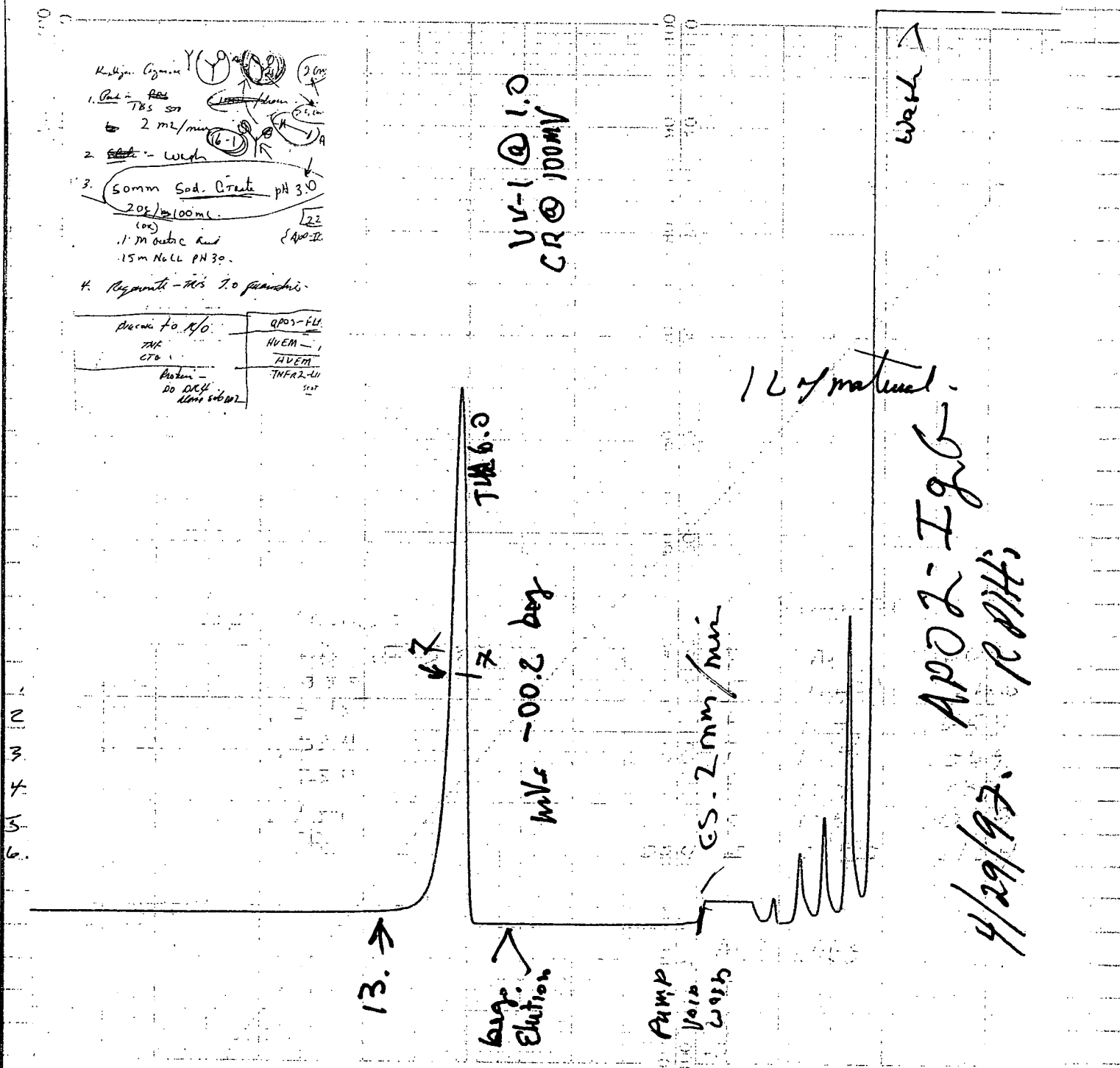
Invented by 

Date

Recorded by

7/29/93

0806/N50/24726/9.90



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**Date**

**Invented by**

Recorded by

Date: \_\_\_\_\_

**To Page No.**

From Page No. —

BSA STD Curve.

1	0	0	4	N	0	mg/ml
2	.016	15.75	5	N	.014	0
3	.039	31.25	6	1:5	.571	3.179 mg
4	.069	62.5	7	1:5	.400	2.175 mg
5	.153	125	8	1:5	.085	.326 mg
6	.294	250	9	N	.109	.093
7	.517	500	10	N	.030	1
8	.837	1000	11	N	.011	0
			12	N	.008	0
			13	N	.001	0
			14	N	0	0
			15	N	.014	0
			16	N	0	0

DRY 1:50 .061

1.86 mg/mL

$$y = m_0 + m_1 * x$$

$$m_0 = .0293357 \quad 5)$$

$$m_1 = -.0008518$$

$$x = \frac{y - m_0}{m_1} \quad \text{or} \quad \frac{y - .029}{.0085}$$

Total yield

 3.179  
 2.175  
 .326

5.68 mg.

total. 2.68 mg/mL.

Tot 10-10

2.68 mg/mL

in - 20

To Page No. —

Witnessed &amp; Understood by me,

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Recorded by

Date

4/29/92

TITLE

125 I-AP02L binding to AP02-IgG+

AP02 FLAG

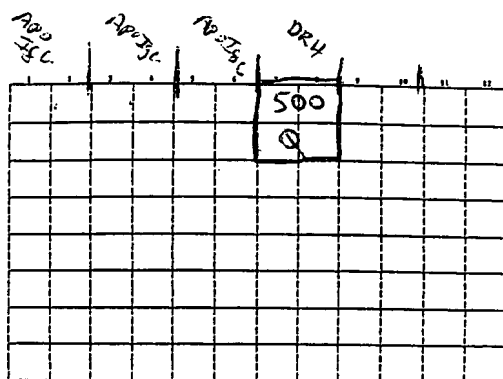
Project No.

Book No. 27510

17

From Page No. \_\_\_

Response: to no



AP02-IgG @  
1 mg/ml.

$\alpha$ FLAG 3 mg/ml 1:600 8.3  $\mu$ l into 5 mL

Goat  $\alpha$ FC 1 mg/ml 1:200 25  $\mu$ l into 5 mL

A. Dilution of AP02L cold. Stock is 38 nM  
1:40 2X is 500 nM (final)

25  $\mu$ l of cold needed per each 3 fold.

12.5 + 488  $\xrightarrow[1:3]{125}$  125 + 250 etc.

B. Dilution of Hot 3.9 x 10<sup>8</sup> cpm/well.  
1:200 dilution for 2X @ array vol. of 5  $\mu$ l

7.6  $\mu$ l into 1.5 mL

To Page No. 235

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Invented by

Date

Recorded by

4/29/97



From Page No. \_\_\_\_\_

Concise to lactoperoxidase label ap02L

A. AP02L 840ug/ml or 38NM

$$\frac{38182 \text{ NMoles}}{L} \times \frac{L}{10^3 \text{ ml}} = \frac{38 \text{ NM}}{\text{ml}}$$

$$38 \text{ NMoles/ml} \text{ or } .038 \text{ NMoles/ml}$$

$$\frac{840 \text{ ug}}{\text{ml}} \text{ or } \frac{38 \text{ NMoles}}{L} \times \frac{L}{10^6 \text{ ul}} = .038 \text{ NMoles/ml}$$

6.6 Only 840ug/ml material is .25 NMoles.

B See following page for isolation procedure  
& calculation

To Page No. \_\_\_\_\_

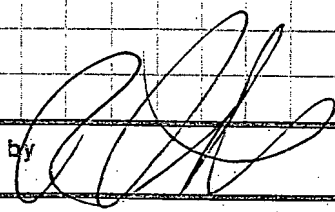
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5/1/97

From Page No. \_\_\_\_\_

## Lactoperoxidase Iodination with PD-10

## Materials and Reagents:

Protein: AP02 L  
 0.4 M Na Acetate, pH 5.6  
 0.1 M Na Acetate, pH 5.6  
 Na <sup>125</sup>I: Amersham IMS.30 (Calibration date 4/28; Decay factor \_\_\_\_\_)  
 Lactoperoxidase: 23582-90 (Calbiochem 427 488): 100 IU/mL  
 30% H<sub>2</sub>O<sub>2</sub>: Sigma H 1009  
 Water for Irrigation (WFI)  
 Sephadex G-25M PD-10 Column: Pharmacia 17-0851-07  
 Column Buffer: ☒ PBS/0.5% BSA  
                   ☐ PBS/0.5% BSA/0.05% Tween 20  
                   ☐ PBS/0.5% BSA/0.05% Tween 20/1 M NaCl  
                   ☐ PBS/0.05% Tween 20

50% TCA

## Procedure:

Pipet 5.5  $\mu$ g/ 6.6  $\mu$ L (0.25 nmol) protein into 1.5 mL microfuge tube  
 Add 15  $\mu$ L 0.4 M Na Acetate, pH 5.6  
 Add 0.52 mCi/ 5  $\mu$ L (0.25 nmol) Na <sup>125</sup>I (0.5 nmol/mCi on calibration date)  
 Add 5 mIU/ 5  $\mu$ L Lactoperoxidase (Dilute 100 IU/mL stock serially:  
 1: 100, ie, 2  $\mu$ L + 198  $\mu$ L and 1: \_\_\_\_\_, ie, \_\_\_\_\_  $\mu$ L + \_\_\_\_\_  $\mu$ L  
 • in 0.1 M Na Acetate, pH 5.6 to \_\_\_\_\_ mIU/mL)  
 Add 5.5 ng/ 5  $\mu$ L (0.25 nmol) H<sub>2</sub>O<sub>2</sub>. (Dilute 300 mg/mL (8.7 mol/L) stock serially:  
 \_\_\_\_\_ + 100, ie, 2  $\mu$ L + 198  $\mu$ L and 1: 34.8, ie, 2  $\mu$ L + 67.6  $\mu$ L  
 in WFI to 0.56  $\mu$ g/mL (25  $\mu$ mol/L))  
 Incubate 5 min. at ambient temp. with intermittent vortexing.  
 Add 5.5 ng/ 5  $\mu$ L (0.25 nmol) H<sub>2</sub>O<sub>2</sub>.  
 Incubate 5 min. at ambient temp. with intermittent vortexing.  
 Quench reaction by adding \_\_\_\_\_  $\mu$ L WFI. Sub TCA + 9.5 300 WFI  
 Total volume: \_\_\_\_\_  $\mu$ L.  
 Pipet 5  $\mu$ L aliquot for TCA precipitation.  
 Transfer iodination mixture to PD-10 column (equilibrated with 25 ml column buffer) and  
 allow to run into gel bed.  
 Add \_\_\_\_\_ ml column buffer (total added = 2.5 mL).  
 Collect and discard 2.5 mL from column.  
 Add 3.5 mL column buffer.  
 Collect \_\_\_\_\_ x \_\_\_\_\_ mL fractions  
 Count 5  $\mu$ L aliquots from peak fractions in gamma counter.  
 Pool peak fractions: \_\_\_\_\_  
 Pipet 5  $\mu$ L aliquot for activity and for TCA precipitation.

2 pulses

Cite  
laet.

That's correct

Lactoperox  
 Stock  
 100 mL dilute 1:100

## 6. Calculations

a.  $[1 - (C: 123605 / A: 485854)] = E: .745$  Decimal fraction  
 Incorporation

b.  $[1 - (D: 26311 / B: 123605)] = .787$  Decimal fraction  
 Precipitability of Pool

c. Specific Activity (uci/ug) =  $\frac{5000 \text{ uCi used} \times E: .745}{372.5 \text{ uCi} \times 5.5 \text{ ug protein used}} = F: .677$  uCi/ug

d. cpm/mL = B:  $\frac{123605 \times 50}{5000} = G: 6.18 \times 10^6$

e. ug/mL = G:  $\frac{6.18 \times 10^6}{F: .677} \div 2.12 \times 10^6 = H: .043$  ug/mL

f. Protein recovered = H:  $.043 \times 2 \text{ mL vol of pool} = I: .086$  ug yield.

g. % Protein recovered = I:  $.086 / 5.5 \text{ ug protein used} \times 100 = 3.2\%$

e. Lot Number \_\_\_\_\_ f. Date of iodination 5/1/97

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Recorded by

AP02-IgT / AP02LI<sup>125</sup>

Project No. \_\_\_\_\_  
Book No. 27510

23

TITLE

Bending Assay

From Page No. \_\_\_\_\_

I. Purpose: to Bind AP02LI<sup>125</sup> to AP02-IgT in vitro

A. AP02LI<sup>125</sup>

HOT Dilute 1:15 in assay buffer

final concentration is 65 p.molar.

B. Cold n. 38  $\mu$ M (monomer)

dilute 2x to 4.00 nM then serially 1:3

10 + 990  $\xrightarrow{1:3}$  100 + 200 etc.

Thimer

66.6

To Page No. \_\_\_\_\_

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Date

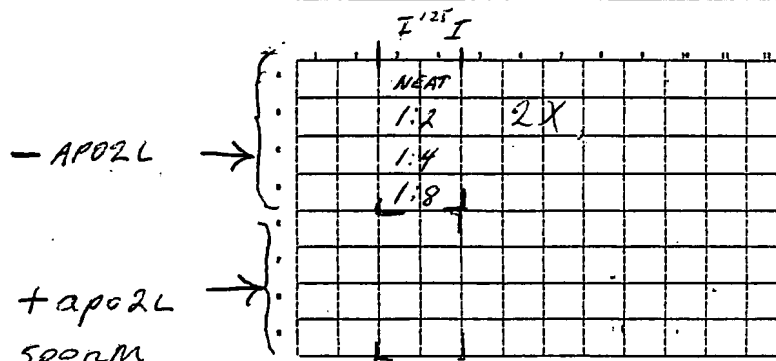
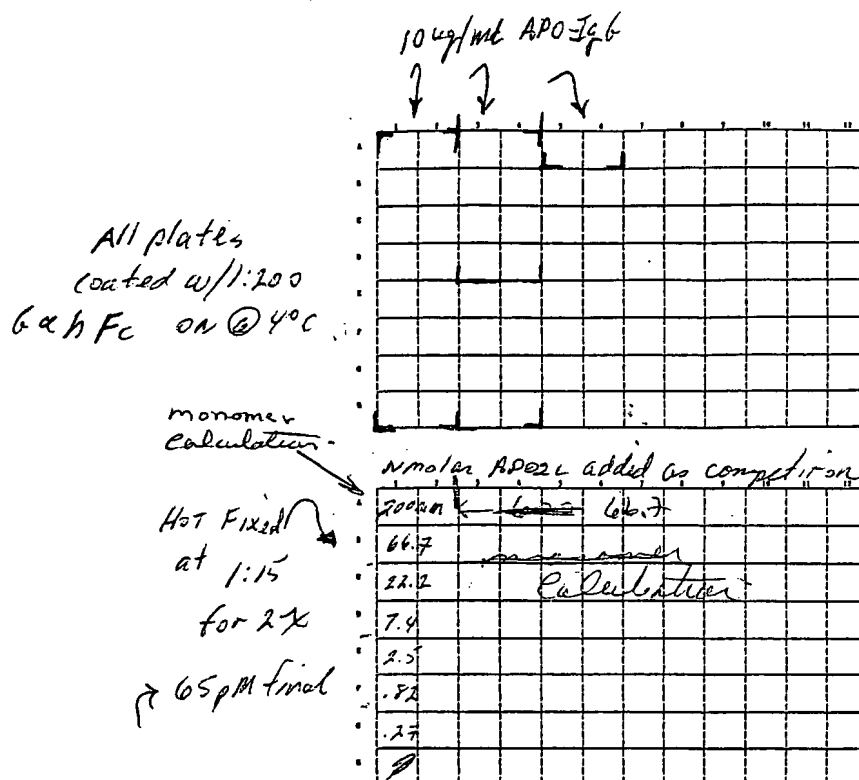
Invented by

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3/1/97

From Page No. \_\_\_\_\_



To Page No. \_\_\_\_\_

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Date

Invented by

Recorded by

Date

9/3/97

TITLE AP02-IgG / AP02L<sup>125</sup>I Binding

Project No. \_\_\_\_\_  
Book No. 27510

25

From

May 01 1997 Report for CPM			
Group/Ch	Tray/Tube	CPM	CPM XCV
1 A	101	1692	0
2 A	102	1553	0
3 A	103	2471	0
4 A	104	2398	0
5 A	105	3027	0
6 A	106	2690	0
7 A	107	3105	0
8 A	108	2798	0
9 A	109	2948	0
10 A	110	3067	0
11 A	111	2658	0
12 A	112	2745	0
13 A	113	2695	0
14 A	114	2905	0
15 A	115	2668	0
16 A	116	2437	0
17 A	117	35625	0
18 A	118	34176	0
19 A	119	18741	0
20 A	120	17638	0
21 A	201	8658	0
22 A	202	8389	0
23 A	203	4148	0
24 A	204	4215	0
25 A	205	9872	0
26 A	206	12147	0
27 A	207	6288	0

Array buffer pH 7.5 PBS/  
1% BSA

Procedure:

1. Remove a-well maxisorb plates  
were coated @ 1:200 dilution  
in 50mm carbonate buffer  
O/N @ 4°C with

Goat- $\lambda$ -human-FC  
Buehring Mannheim

2. plates were washed 3x in  
0.5% Tween PBS and blocked  
for 1 hour with array buffer

3. plates were then loaded with  
1  $\mu$ g/ml AP02-IgG for  
1 hour @ RT in the plate-shaker

4. plates were washed 3x in  
wash buffer and  
label & cold were added  
in final and concentration  
shown on B.24.

5. Plate was washed 5x with  
wash buffer and then counted.

May 01 1997 Report for CPM			
Group/Ch	Tray/Tube	CPM	CPM XCV
28 A	208	6440	0
29 A	209	2969	0
30 A	210	2513	0
31 A	211	1785	0
32 A	212	1701	0
33 A	213	4	0
34 A	214	0	0
35 A	215	0	0
36 A	216	0	0
37 A	217	4	0
38 A	218	0	0

Witnessed & Understood by me,

Date

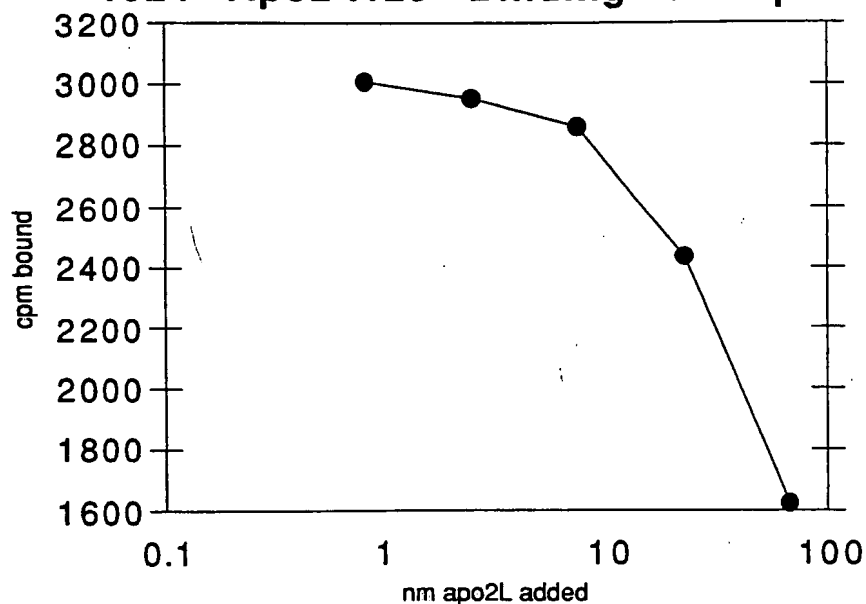
Invented by

Date

To Page No. \_\_\_\_\_

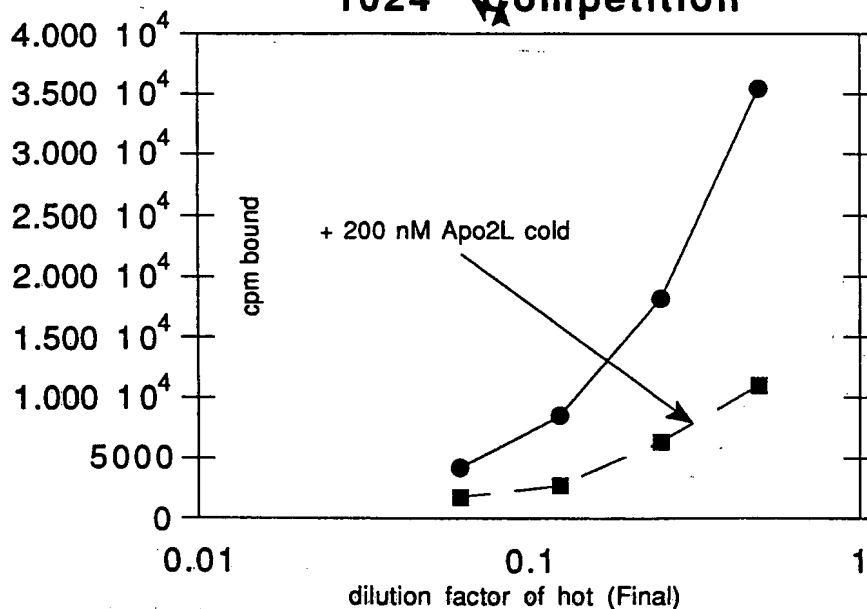
Recorded by

From Page No. \_\_\_\_\_

**1024 Apo2-I125 Binding to Apo2-IgG**

age #1 - "Data 24"

	MEAN	STDEV	C
1	1623.0	98.995	66.700
2	2434.5	51.619	22.200
3	2858.5	238.29	7.4000
4	2952.0	217.79	2.5000
5	3007.5	84.146	0.82000

**1024 Competition**

Witnessed &amp; Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by RAHDate 5/1/97

Recorded by \_\_\_\_\_

To Page No. \_\_\_\_\_

TITLE

Biacore

Project No. \_\_\_\_\_

Book No. 27510

27

From Page No. \_\_\_\_\_

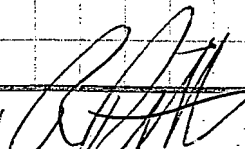
Purpose: to measure a  $K_D$  for APO2L  
binding to F199-APO. by Biacore analysis.

Dilution of APO2L material was spun  
and assayed by Bradford prior to  
analysis - (10% loss after spin -

Witnessed &amp; Understood by me,

Date

Invented by



Date

2/2/97

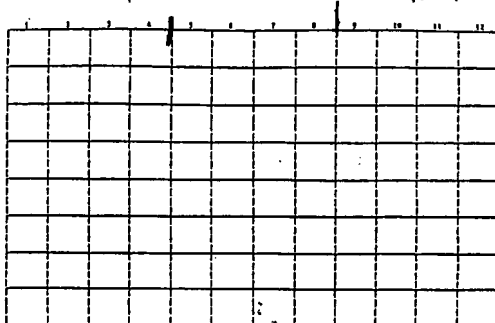
Recorded by

To Page No. \_\_\_\_\_

From Page No. \_\_\_\_\_

Assay Buffer PBS/1% BSA pH 7.5.

1000 µg/ml APO2-IgG  
 Receptor      No Receptor



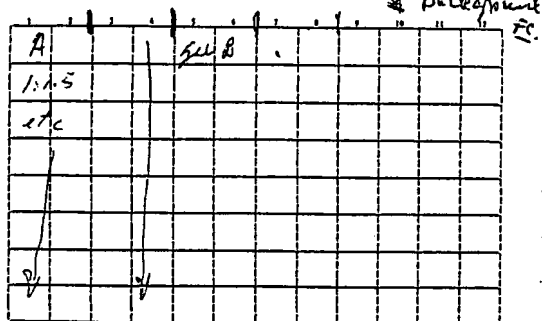
Saturation  
 Curve

mix 50:50

A) undiluted label

B) 100 µl C.W.

C) Dilute serially  
 1:1.5



Calculations

A. Saturation

400 µl of neat label + 400 µl of 1:40 (of 840 µg/ml APO2L)  
 serially diluted 1:1.5

400 I<sup>125</sup>-APO2L + 400 µl in 40 dilution → 500 + 250 etc

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Recorded by



From Page No. \_\_\_\_\_

B. Calculations for Competition

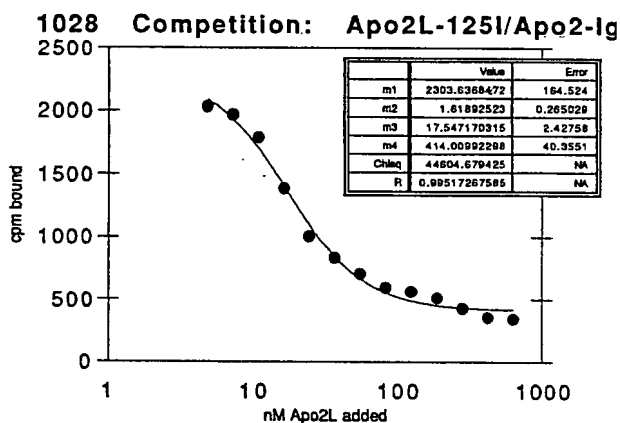
1. Dilute hot for 2x @ 1:20

2. Dilute cold

1:10 then Serially 1:1.5

80 (1/5 stock 84 angstrom Apo2L) + 720 assay buffer  $\xrightarrow{1:1.5}$

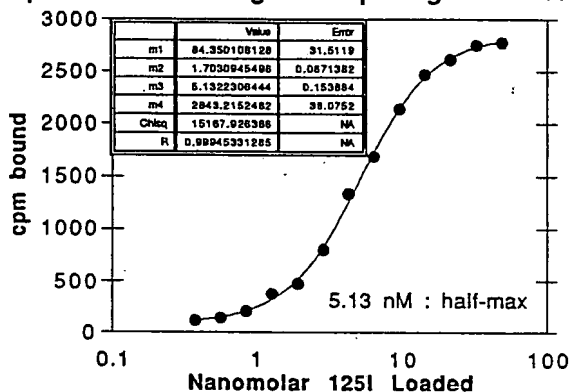
500 + 250 etc



age #1 - "1028data"

	MEAN	STDEV	C
1	345.00	31.113	636.00
2	359.50	9.1924	423.96
3	429.50	21.920	282.61
4	511.00	52.326	188.39
5	561.50	9.1924	125.58
6	593.00	52.326	83.711
7	705.50	21.920	55.802
8	834.00	8.4853	37.198
9	1003.0	241.83	24.796
10	1384.0	4.2426	16.529
11	1788.5	0.70711	11.018
12	1969.0	22.627	7.3447
13	2032.0	42.426	4.8960
14	2024.5	129.40	3.2637
15	1919.0	45.255	2.1756
16	1657.5	24.749	1.4502

Apo2-I125 Binding to Apo2-IgG: Saturation



age #1 - "1028apodata"

	MEAN	STDEV	C	MEAN	STDEV	F	G
1	6487.0	206.06	500.00	1155.5	235.7	5331.5	
2	5410.5	300.52	333.30	996.00	226.7	4414.5	
3	3772.5	395.27	222.18	478.00	33.941	3293.5	
4	3056.0	11.314	148.10	282.00	42.476	2774.0	49.000
5	2968.0	267.29	98.726	219.50	6.31	2748.5	32.663
6	2815.0	9.8995	65.811	205.50	41.719	2609.5	21.773
7	2589.0	80.610	43.869	122.50	19.092	2466.5	14.514
8	2213.0	46.669	29.243	77.000	65.054	2136.0	9.6751
9	1782.0	107.48	19.494	98.500	62.933	1683.5	6.4494
10	1385.0	76.368	12.994	52.500	18.092	1332.5	4.2992
11	863.00	18.385	8.6621	64.000	29.698	799.00	2.8658
12	580.00	57.983	5.7742	108.50	109.60	471.50	1.9104
13	421.00	22.627	3.8491	47.500	17.678	373.50	1.2735
14	254.00	15.556	2.5658	51.000	31.113	203.00	0.84889
15	169.50	12.021	1.7103	30.000	26.870	139.50	0.56587
16	124.00	1.4142	1.1401	10.000	8.4853	114.00	0.37721

Witnessed & Understood by me,

Date

Invented by

Date

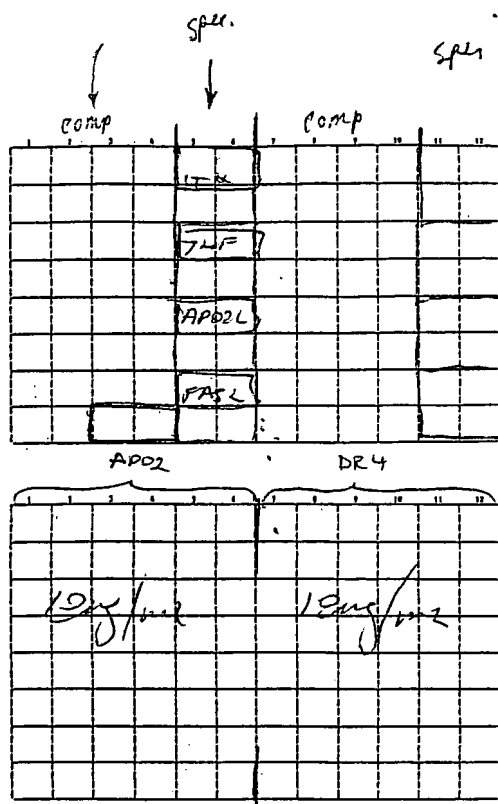
To Page No. \_\_\_\_\_

Recorded by

From Page No. \_\_\_\_

Assay buffer is pH 6.5 PBS / 1% BSA -

For dilutions of LT $\alpha$ , TNF $\alpha$ , FasL, + APO2L in  
specificity data see p. 14.



LT $\alpha$	1:15	12 $\mu$ L + 140
TNF $\alpha$	1:75	2 $\mu$ L + 138
APO2L	1:100	1.5 $\mu$ L + 148.5
FasL	1:75	20 $\mu$ L + 130

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Date

Invented by

Date

To Page No. \_\_\_\_

Recorded by

5/6/97



From Page No. \_\_\_\_\_

## II. Procedure

## A. Calculations

1. Cost @ 1:200 2FC
2. Both APO-IgG and DR4-IgG are both added  
at 20  $\mu$ g/ml

DR4-IgG 1480  $\mu$ g/ml 1:75 40 + 2960APO2-IgG 2680  $\mu$ g/ml 1:130 23 + 2977

3. Concentration curve

Witnessed &amp; Understood by me:

Date

Invented by

Recorded by

To Page No. \_\_\_\_\_

Date

5/7/97

From Page No. \_\_\_\_\_

Direct Coupling

AP22-IG6

$$\frac{2680}{50} = 1:50 \text{ dilution}$$

PR4-IG6

$$\frac{1480}{50} = 1:30 \text{ dilution}$$

5/8 FCY

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Recorded by

5/5/92

1:200 6045  $\alpha$  FC Coat in Carbonate

5 mg/L  
10 mg/L  
20 mg/L } Birth receptors

[illegible]

Legend 50ug/ml 2x and then serially 1:2

Calculation of molarity, based on 66 kD value

Date \_\_\_\_\_

Recorded by

ate 7/8/97

TITLE Solid Phase Binding

Project No. \_\_\_\_\_  
Book No. 27510

39

From Page No. \_\_\_\_\_

Page #1 - "Data 1"

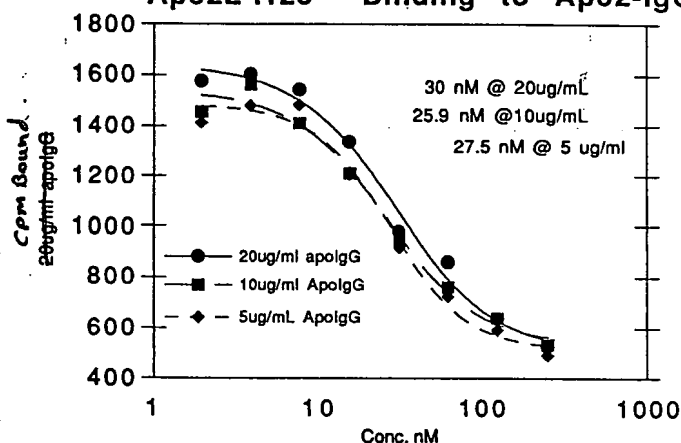
Friday, May 9 3:08 AM

	20ug/ml apolG	STDEV	10ug/ml ApolG	STDEV	5ug/ml ApolG	STDEV	20ug/mL DR4
1	527.00	62.225	535.00	33.941	492.00	8.4853	367.00
2	638.00	67.882	639.00	15.556	592.50	21.920	473.00
3	860.00	25.456	761.00	7.0711	725.50	23.335	571.50
4	979.00	63.640	946.50	64.347	917.50	61.518	785.50
5	1337.0	83.439	1209.5	28.991	1204.5	103.94	1019.0
6	1544.5	103.94	1412.0	28.284	1485.5	14.849	1270.5
7	1603.5	28.991	1566.5	70.004	1481.5	16.263	1413.0
8	1578.0	50.912	1457.0	41.012	1414.0	50.912	1288.5

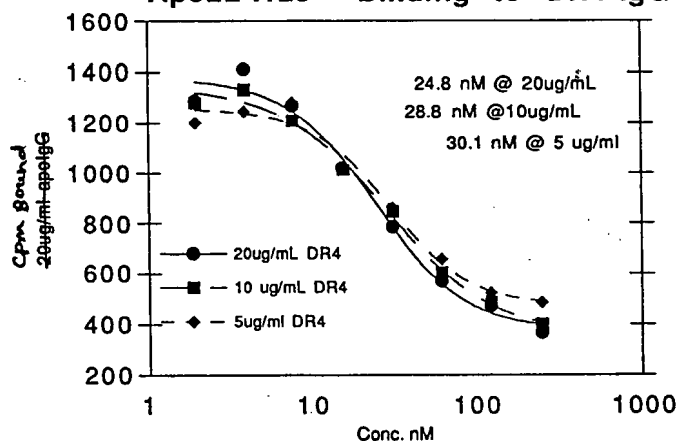
Page #2 - "Data 1"

	STDEV	10 ug/mL DR4	STDEV	5ug/ml DR4	STDEV	Conc. nM	N
1	39.598	400.50	20.506	487.00	29.698	250.00	
2	9.8995	488.00	5.6569	524.50	0.70711	125.00	
3	7.7782	605.50	6.3640	658.00	9.8995	62.500	
4	43.134	845.00	52.326	858.00	33.941	31.250	
5	69.296	1013.0	46.669	1020.5	64.347	15.625	
6	23.335	1210.0	100.41	1282.0	14.142	7.8125	
7	21.213	1334.5	28.991	1248.5	36.062	3.9062	
8	68.589	1282.0	73.539	1201.5	19.092	1.9531	

1038 Competition Curves for Apo2L-I125 Binding to Apo2-IgG



1038 Competition Curves for Apo2L-I125 Binding to DR4-IgG



Witnessed & Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

To Page No. \_\_\_\_\_

From Page No. —

Genproc. to determine KD of APO2L to APO-196

Lyzand. BLM - 59aabnd

Inst. Dilution is 1:12.5 of 842 ug/ml -

APO2L f.c. to \* 4 (file)

f.h. is 12 ug/ml

ing vol is 30

Bulker. 0.05% JW / PBS PH 7.5.

1:12.5  $\xrightarrow{1:2}$  1:25  $\xrightarrow{1:2}$  1:50 etc Lyand  
for eight pts =

Run on FC 4 = 898 RU APO2-196

Witnessed &amp; Understood by me,

Date

Invented by

Date

To Page No. —

Recorded by

5/9/92



BIACORE

Project No. \_\_\_\_\_  
Book No. 27510

41

TITLE APD2L in an AP-FC

From Page No. \_\_\_\_\_

This is essentially a repeat of the previous.

APD2L 8424y hnt

- |           |   |                        |
|-----------|---|------------------------|
| 1. 1:12.5 | } | good                   |
| 2. 1:25.0 |   |                        |
| 3. 1:50   | } | inverted               |
| 4. 1:100  |   |                        |
| 5. 1:200  | } | (Trashed (air bubble)) |
| 6. 1:400  |   |                        |
| 7. 1:800  |   |                        |
| 8. 1:1600 |   |                        |

FC 4 -

511a bnd  $f = 160.40 \text{ m}^{-1}$  -105.

Witnessed & Understood by me,

Date

Invented by

Date

To Page No. \_\_\_\_\_

Recorded by

3/1/97

From Page No. \_\_\_\_\_

FC4 again AP02 IgG  $\approx$  900 Rn -

1 Q (Butter) -  
2 1:800  
3 1:400  
4 1:200  
5 1:100  
6 1:50  
7 1:25  
8 1:12.5  
~~9~~

Witnessed &amp; Understood by me,

Date

Invented by

Date

Recorded by

To Page No. \_\_\_\_\_

5/12/97

NOTEBOOK NO. 26508  
ISSUED TO JAME P. SHERIDAN  
ON [REDACTED] 19 [REDACTED]  
DEPARTMENT MOLECULAR ONCOLOGY  
RETURNED 19

—SCIENTIFIC NOTEBOOK CO.—  
2831 LAWRENCE AVE.  
P.O. BOX 238  
STEVENSVILLE, MI 49127  
616-429-8285

ADE- 22  
USSN 10/052,798

455 G TO  
CAP S TO  
USB S TO  
SAVO

Project No. \_\_\_\_\_  
Book No. 26508 TITLE \_\_\_\_\_

From Page No. \_\_\_\_\_

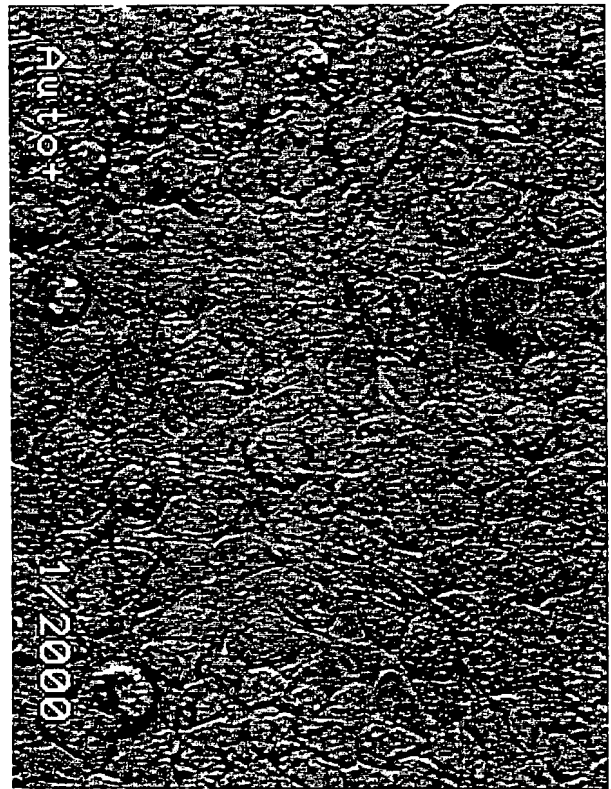
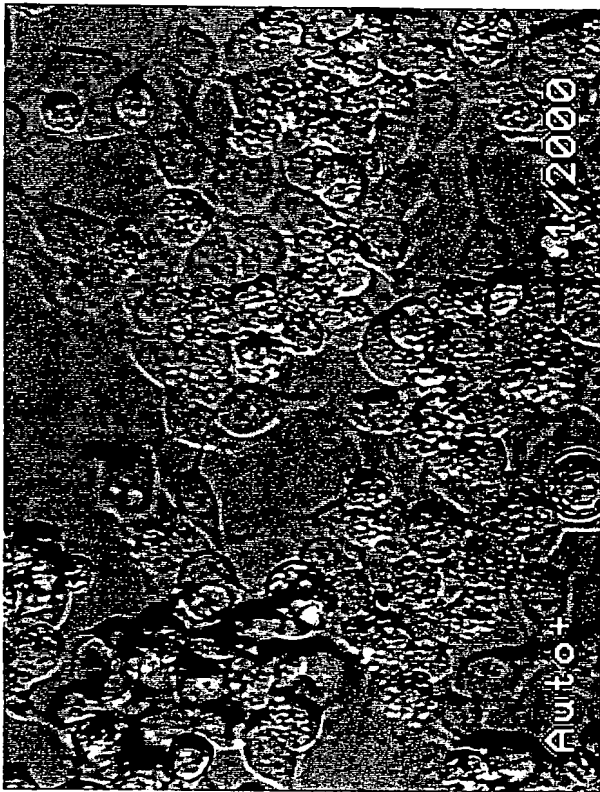
PHOTOGRAPHED APOPTOSIS INDUCED BY OVEREXPRESSION OF  
DONTIN DONTIN CONTAINING PLASMA FROM THE ~~LAST~~ PROJECT.  
IN CYC DATABASIS.

1. CONTROL @ LOW MAG. (COLOR GREEN)
2. TRANSFECTED (APOPTOTIC) @ HIGH MAG. (COLOR GREEN)
3. TRANSFECTED (APOPTOTIC) @ HIGH MAG. (BLACK AND WHITE)

1. DD - 293 - [REDACTED]

4. CONTROL PRKS

2. PRKS + 293 - PRKS - [REDACTED]



To Page No. \_\_\_\_\_

Witnessed & Endorsed by me,

rented by

*Joseph S. Schuch*

Date

[REDACTED]

Recorded by

*Jan P. Schuch*

Fri

293 cells

- I) 500K cells for FACS  
 II) 500K cells for DNA Ladder  
 III) 1000K cells for NFkB  
 IV) 3000K cells for SAP/cJun

○ = PAUSETED  
 AFTER 24 HRS.  
 SAUO AS IS

USUAL  
 24 HRS

- |     |                     |   |                   |            |
|-----|---------------------|---|-------------------|------------|
| 1.  | pRK5 (20µg/ml)      |   |                   |            |
| 2.  | pRK5 (20µg/ml)      |   |                   |            |
| 3.  | 2-1 (10µg/ml)       | + | pRK5 (10µg/ml)    |            |
| 4.  | 2-1 (10µg/ml)       | + | pRK5 (10µg/ml)    |            |
| 5.  | 2-1 (10µg/ml)       | + | CRMA (10µg/ml)    |            |
| 6.  | 2-1 (10µg/ml)       | + | CRMA (10µg/ml)    |            |
| 7.  | 2-1 (10µg/ml)       | + | FADD-DN (10µg/ml) |            |
| 8.  | 2-1 (10µg/ml)       | + | FADD-DN (10µg/ml) |            |
| 9.  | 2-1 (10µg/ml)       | + | pRK5 (10µg/ml)    | + DEVD-Fmk |
| 10. | 2-1 (10µg/ml)       | + | pRK5 (10µg/ml)    | + DEVD-Fmk |
| 11. | pRKCRMA (10µg/ml) + |   | pRK5 (10µg/ml)    |            |
| 12. | pRKCRMA (10µg/ml) + |   | pRK5 (10µg/ml)    |            |

BLOCKED

MYST  
BLOCKED

BLOCKED

Add the DEVD-Fmk when the medium is changed.

To Page No. \_\_\_\_\_

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Date

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## TITLE

## INCYTE constructs

Scot Marsters obtained possible clones of death domain containing constructs using the INCYTE Data Base on last Friday.

Step 1 (Tuesday 11PM) Electroporate 1,000K HeLa cells with 8µg of gene X, 8µg of gene pRK5, 1µl VARNA, and 4µg of pRKCD4.

Step 2 change medium 11PM

Step 3 Express for 24hrs and harvest for FACS. 4PM WOODS DAY

✓ pRK5 (16µg/ml) 1  
✓ pRK5 (8µg/ml) 11µl 1-1 (8µg/ml) 2  
pRK5 (8µg/ml) 6µl 2-1 (8µg/ml) 3  
pRK5 (8µg/ml) 5µl 3-1 (8µg/ml) 4  
pRK5 (8µg/ml) 5µl 4-1 (8µg/ml) 5

(+) pRKCD4 6  
(+) pRK5 7  
(-) pRKCD4 (+) pRKCD4 8

✓ pRK5 (8µg/ml) + CRMA (8µg/ml)  
1-1 (8µg/ml) + CRMA  
2-1 (8µg/ml) + CRMA  
3-1 (8µg/ml) + CRMA  
4-1 (8µg/ml) + CRMA

2-1 (8µg/ml) + MuFADD-DN (8µg/ml)  
2-1 (8µg/ml) + HuFADD-DN (8µg/ml)  
2-1 (8µg/ml) + Hu-TRADD-DN (8µg/ml)  
2-1 (8µg/ml) + Hu-TRAF2-DN (8µg/ml)

Add DEVD 4hrs after electroporation

pRK5 (16µg/ml) + DEVD  
pRK5 (8µg/ml) 2-1 (8µg/ml) + DEVD

Add ALLN (100ng/ml) 4hrs before harvest

pRK5 (16µg/ml) + ALLN  
pRK5 (8µg/ml) 2-1 (8µg/ml) + ALLN

PE 15 ALONG  
FITC FITC ALONG  
PE PE + FITC

DID NOT  
DO

DID NOT  
DO

20  
21 FORGET TO DO  
#21

## ANALYSIS RESULTS

% FITC

POSITIVES

SAMPLE # % PG POSIT. % FITC

#1 12.1% 14.1%

#3 30.6% 35.4%

#9 10.9% 12.9%

#11 10.1% 11.4%

#14 22.8% 48.5%

#15 28.9%

#16 42.5%

#17 50.8%

#18

#19 54.4% 60.1%

#20 23.2%

23 x 0.4ml = 9.2ml

23 x 10µl PG = 230µl PG

207 230µl: 23µl AMIXIN

CONCLUSION: OVEREXPRESSION OF 2-1 CAUSED APOPTOSIS  
- THIS COULD BE BLOCKED BY CRMA  
- THIS COULD BE REDUCED BY HuFADD-DN

NOTE: THE PG STAINING WAS OKAY. THE FITC STAINING WAS WEAK. INCREASE THE FITC BY 100% NEXT TIME

To Page No.

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Date

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From Page No.     

cyclohexamide:HeLa

HeLa:Apo2L, TNF, and LT (plated in HGD MEM)

Dose response in the presence and absence of cyclohexamide Plate ~200K cells in 6 well plates on the previous night. Cells will be pretreated with ALLN for 1 hr prior to Apo2L. Cells will be incubated with Apo2L for 5hrs prior to annexin staining.

1:00AM pretreat 12 wells +/- cyc. @ 40µg/ml (1:1000) for 1hr.

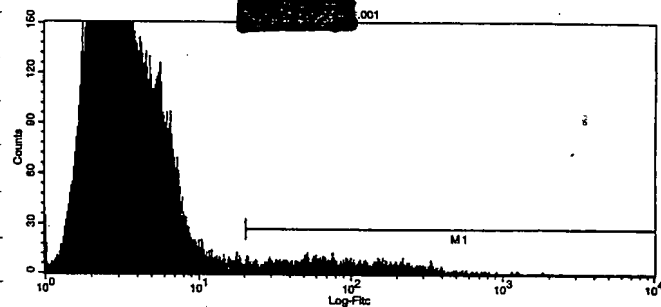
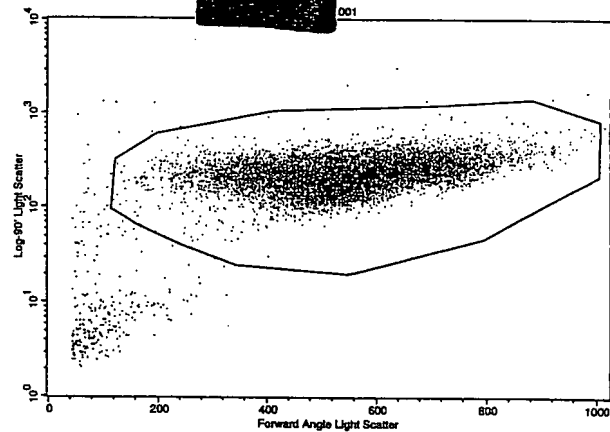
1:00AM treat cells with each ligand @ 1µg/ml.

4:00PM Harvest and annexin stain (no PI).

Apo2L				TNF				LT(lymphotoxin)			
37	cyc +	NT	49	cyc +	NT	61	cyc +	NT			
38	cyc +	1:500	50	cyc +	1:500	62	cyc +	1:100			
39	cyc +	1:1000	51	cyc +	1:1000	63	cyc +	1:200			
40	cyc +	1:2000	52	cyc +	1:2000	64	cyc +	1:400			
41	cyc +	1:4000	53	cyc +	1:4000	65	cyc +	1:800			
42	cyc +	1:8000	54	cyc +	1:8000	66	cyc +	1:1600			
43		NT	55		NT	67		NT			
44		1:500	56		1:500	68		1:100			
45		1:1000	57		1:1000	69		1:200			
46		1:2000	58		1:2000	70		1:400			
47		1:4000	59		1:4000	71		1:800			
48		1:8000	60		1:8000	72		1:1600			

SAVED FILES AS!

JSA

Sample ID: 37  
Total Events: 30880

Gated Events: 29953

Marker	% Gated
All	100.00
M1	5.35

Apo2L				TNF				LT			
37	5.4%			49	4.7%			61	8.1%		
38	8.2%			50	16.0%			62	16.3%		
39	67.6%			51	35.6%			63	14.3%		
40	59.1%			52	31.9%			64	16.9%		
41	54.9%			53	13.0%			65	8.3%		
42	42.7%			54	17.4%			66	6.8%		
43	5.0%			55	7.5%			67	4.2%		
44	35.8%			56	9.8%			68	5.5%		
45	34.6%			57	5.1%			69	4.8%		
46	25.3%			58	8.1%			70	5.2%		
47	16.3%			59	8.2%			71	5.9%		
48	5.1%			60	6.6%			72	5.4%		

To Page No.     

Witnessed &amp; Understood by me,

Date

Invented by

Date

Recorded by

F

## ALLN:HeLa

HeLa:Apo2L, TNF, and LT (plated in HGMEM)

Dose response in the presence and absence of ALLN (inhibitor of IkB degradation). Plate ~200K cells in 6 well plates on the previous night. Cells will be pretreated with ALLN for 1 hr prior to Apo2L. Cells will be incubated with Apo2L for 5hrs prior to annexin staining.

8:00AM pretreat 12 wells +/- ALLN @ 40µg/ml (1:1000) for 1hr.

9:00AM treat cells with each ligand @ 1µg/ml.

2:00PM Harvest and annexin stain (no PI).

	293 Apo2L	293 TNF	293 LT(lymphotoxin)
1	ALLN + NT 13	ALLN + NT 25	ALLN + NT
2	ALLN + 1:500 14	ALLN + 1:500 26	ALLN + 1:100
3	ALLN + 1:1000 15	ALLN + 1:1000 27	ALLN + 1:200
4	ALLN + 1:2000 16	ALLN + 1:2000 28	ALLN + 1:400
5	ALLN + 1:4000 17	ALLN + 1:4000 29	ALLN + 1:800
6	ALLN + 1:8000 18	ALLN + 1:8000 30	ALLN + 1:1600
7	NT 19	NT 31	NT
8	1:500 20	1:500 32	1:100
9	1:1000 21	1:1000 33	1:200
10	1:2000 22	1:2000 34	1:400
11	1:4000 23	1:4000 35	1:800
12	1:8000 24	1:8000 36	1:1600

SPON 15

FACS 152

COLLECTING 50,000

GATED 620NTS

Apo2L 4.4 µl in 2 wells  
TNFα 4 µl in 2 wells  
LTβ 20 µl in 2 wells

200K 38 = 7600 K

35 µl = 79 µl

38 x 0.4 µl = 15.2 µl

38 µl = 34.2 µl

1	4.8%	13	5.8%	25	5.6%
2	80.3%	14	12.8%	26	6.2%
3	64.0%	15	10.3%	27	7.1%
4	51.2%	16	8.8%	28	8.4%
5	34.4%	17	9.0%	29	5.7%
6	17.4%	18	7.9%	30	6.4%
7	6.6%	19	6.2%	31	4.8%
8	51.0%	20	7.9%	32	6.5%
9	31.6%	21	8.3%	33	6.0%
10	26.9%	22	6.3%	34	5.5%
11	18.4%	23	6.2%	35	6.1%
12	7.2%	24	7.1%	36	7.1%

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Recorded by



## INCYTE construct 2-1

- Step 1 Electroporate 1,000K HeLa cells with 8 $\mu$ g of gene X, 8 $\mu$ g of gene pRK5,  
1 $\mu$ l VARNA, and 4 $\mu$ g of pRKCD4.  
Step 2 Change medium after 4hrs.  
Step 3 Express for 24hrs and harvest for FACS.

A	(+) pRKCD4		PE
B	(-) pRKCD4	(+) pRK5	PE
C	(+) pRK5 + pRKCD4	+Apo2	FITC + PE

1	pRK5 (16 $\mu$ g/ml)	
2	pRK5 (16 $\mu$ g/ml)	
3	pRK5 (12 $\mu$ g/ml)	2-1 (4 $\mu$ g/ml) 2.9 $\mu$ l
4	pRK5 (12 $\mu$ g/ml)	2-1 (4 $\mu$ g/ml)
5	pRK5 (10 $\mu$ g/ml)	2-1 (6 $\mu$ g/ml) 4.3 $\mu$ l
6	pRK5 (10 $\mu$ g/ml)	2-1 (6 $\mu$ g/ml)
7	pRK5 (8 $\mu$ g/ml)	2-1 (8 $\mu$ g/ml) 5.7 $\mu$ l
8	pRK5 (8 $\mu$ g/ml)	2-1 (8 $\mu$ g/ml)
9	pRK5 (6 $\mu$ g/ml)	2-1 (10 $\mu$ g/ml) 7.1 $\mu$ l
10	pRK5 (6 $\mu$ g/ml)	2-1 (10 $\mu$ g/ml)
11	pRK5 (4 $\mu$ g/ml)	2-1 (12 $\mu$ g/ml) 8.6 $\mu$ l
12	pRK5 (4 $\mu$ g/ml)	2-1 (12 $\mu$ g/ml)
13	2-1 (16 $\mu$ g/ml)	
14	2-1 (16 $\mu$ g/ml) 11.4 $\mu$ l	

SOME LIPS MUST COLL

PROBLEM WITH #6 ELECTROPORATION.

OVERLY 1 $\mu$ l OF Apo2 @ 1:500 DILUTION FOR FINAL CONCENTRATION  
OF 0.25  $\mu$ g/ml

0.7 $\mu$ l PO  
2 $\mu$ l APO2

## 293 Transfection

Split cells 3 days in advance.

Feed cells at least 1hr before transfection (DNA must sit on cells for 4hrs (8hrs max.)), then change the medium.

Tube A

0.5mls TE (diluted 1:10)

DNA (10 $\mu$ g)1 $\mu$ g VARNA

Mix

Add 50 $\mu$ l of 2.5 M CaCl<sub>2</sub>

Dropwise add Tube A into Tube B. Let sit 10min. Dropwise add onto plates.

Tube B

0.5ml 2X HBSS

Witnessed &amp; Understood by me,

Date

Invented by

Date

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TITLE

Project No. \_\_\_\_\_  
Book No. 26508

89

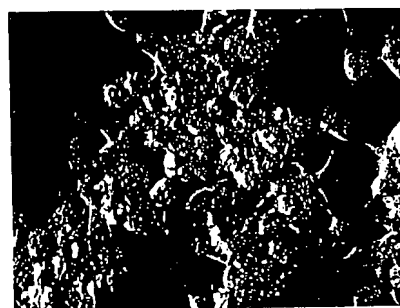
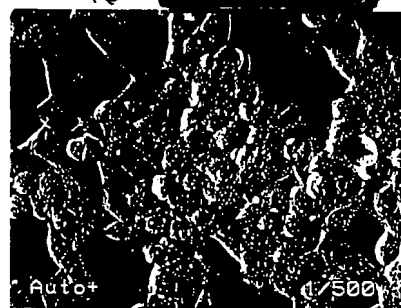
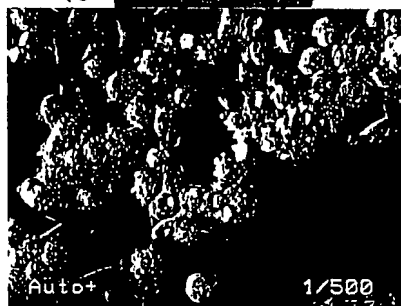
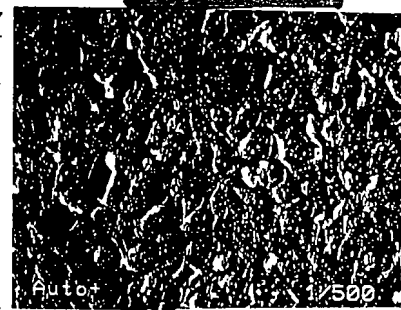
293 cells

1000K cells for DNA Ladder

no 1/500 DNA

- |         |                    |                |  |
|---------|--------------------|----------------|--|
| 1. 0.89 | pRK5 (20µg/ml)     |                | NO APOPTOSIS                           |
| 2. 1.3  | 2-1 (10µg/ml)      | +              | pRK5 (10µg/ml) BLOCK APOPTOSIS         |
| 3. 1.47 | 2-1 (10µg/ml)      | +              | CRMA (10µg/ml) BLOCK APOPTOSIS         |
| 4. 1.54 | 2-1 (10µg/ml)      | +              | HuTRADD-DN (10µg/ml) APOPTOSIS         |
| 5. 0.98 | 2-1 (10µg/ml)      | +              | HuTRAF2-DN (10µg/ml) APOPTOSIS         |
| 6. 0.97 | 2-1 (10µg/ml)      | +              | HuFADD-DN (10µg/ml) NO APOPTOSIS       |
| 7. 0.86 | 2-1 (10µg/ml)      | +              | MuFADD-DN (10µg/ml) APOPTOSIS          |
| 8. 1.04 | 2-1 (10µg/ml)      | +              | pRK5 (10µg/ml) + DEVD-Fmk NO APOPTOSIS |
| 9. 0.68 | pRKCMA (10µg/ml) + | pRK5 (10µg/ml) | NO APOPTOSIS                           |

Add the DEVD-Fmk when the medium is changed.



STORED IMAGES ON ZIP DISK. 20% OF THE CELLS RECOVERED WERE USED TO PREPARE THE APOPTOTIC DNA

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Recorded by

J. S. L.

Fri

Step 1

INCYTE construct 2-1

Electroporate 1,000K HeLa cells with 8µg of gene X, 8µg of gene pRK5,

1µl VARNAs, and 4µg of pRKCD4.

Step 2

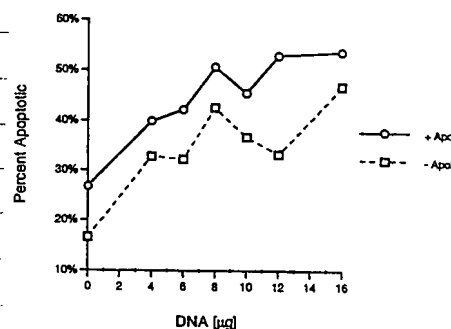
Change medium after 4hrs.

Step 3

Express for 24hrs and harvest for FACS.

A	(+) pRKCD4	(+) pRK5	PE	% Apoptotic
B	(-) pRKCD4		PE	
C	(+) pRK5		FITC + PE	
D	(+) pRK5	- Apoptotic	FITC	
1	pRK5 (16µg/ml)	+Apo2		16.6%
2	pRK5 (16µg/ml)			26.8%
3	pRK5 (12µg/ml)	2-1 (4µg/ml)		32.8%
4	pRK5 (12µg/ml)	2-1 (4µg/ml)		39.9%
5	pRK5 (10µg/ml)	2-1 (6µg/ml)		32.2%
6	pRK5 (10µg/ml)	2-1 (6µg/ml)		42.2%
7	pRK5 (8µg/ml)	2-1 (8µg/ml)		42.6%
8	pRK5 (8µg/ml)	2-1 (8µg/ml)		50.7%
9	pRK5 (6µg/ml)	2-1 (10µg/ml)		36.7%
10	pRK5 (6µg/ml)	2-1 (10µg/ml)		45.5%
11	pRK5 (4µg/ml)	2-1 (12µg/ml)		33.2%
12	pRK5 (4µg/ml)	2-1 (12µg/ml)		52.9%
13	2-1 (16µg/ml)			23.2%
14	2-1 (16µg/ml)			53.6%

HeLa 2-1 transfection +/- 0.0625 µg/ml Apo2L



ADD APO2L @ 11 AM TO THE EVEN #s ONLY O.  
 OVERLAY 1ml OF APO2L @ 1:4000 DILUTION FOR A FINAL  
 CONCENTRATION OF 0.0625 µg/ml.

$$7 \mu\text{L PR5} \times 15 = 105$$

$$20 \mu\text{L AMOXIN} \times 15 = 300 \mu\text{L}$$

$$0.4 \times 135 \times 15 = 6 \mu\text{L}$$

VERY NICE  
STAINING

7 & 20

ON SECOND TROUBLED  
FITC WAS HIGH

TRY 7 & 15  
NEXT TIME

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

From Page No. \_\_\_\_\_

WORKED UP DNA~~W~~ ACCORDING TO PROVIGON PROTOCOL, INCLUDES 1 EXTRA CHLOROFORM EXTRACTION.

SAMPLE	VOLUME AFTER 1:5 WATER DILUTION	H <sub>2</sub> O	10X KLENOW BUFFER	P <sup>32</sup> DILUTED 1:20	KLENOW ENZYME
1	5.6 $\mu$ l	1.4 $\mu$ l	1 $\mu$ l	1 $\mu$ l	1 $\mu$ l
2	2.8 $\mu$ l	4.2 $\mu$ l	↓	↓	↓
3	3.4 $\mu$ l	3.6 $\mu$ l			
4	3.2 $\mu$ l	3.8 $\mu$ l			
5	5.1 $\mu$ l	1.9 $\mu$ l			
6	5.2 $\mu$ l	1.8 $\mu$ l			
7	5.8 $\mu$ l	1.2 $\mu$ l			
8	4.8 $\mu$ l	2.2 $\mu$ l			
9	7.4 $\mu$ l	—			

GB#1

GB#2

RAN 2MS @ 100 VOLTS

1.	8.
2.	6.
3.	7.
4.	4.
5.	5.
6.	4.
7.	5.
8.	4.
9.	5.

USON RADIOS  $\alpha$  <sup>32</sup>P dATP 10  $\mu$ Ci /  $\mu$ l  
DILUTED 1  $\mu$ l into 19  $\mu$ l H<sub>2</sub>O

#4

JS03 PG63

4  $\mu$ l  
NO DILUTION

3  $\mu$ l  
H<sub>2</sub>O

1

1

1

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

Recorded by

From

INCYTE construct 2-1

- Step 1 Electroporate 1,000K HeLa cells with 8μg of gene X, 8μg of gene pRK5.  
1μl VARNA, and 4μg of pRKCD4.  
Step 2 Change medium after 4hrs.  
Step 3 Express for 24hrs and harvest for FACS.

A	(+) pRKCD4			PE
B	(-) pRKCD4	(+) pRK5		PE
C	(+) pRK5			FITC + PE
D	(+) pRK5 + CD4	-apoptosis	+Apo2	+ FITC

1	pRK5 (16μg/ml)	8.6%		
2	pRK5 (16μg/ml)	9.7%		
3	pRK5 (8μg/ml)	29.8%	2-1 (8μg/ml)	
4	pRK5 (8μg/ml)	28.6%	2-1 (8μg/ml)	
5	pRK5 (8μg/ml)	19.3%	+	CRMA (8μg/ml)
6	pRK5 (8μg/ml)	16.6%	+	CRMA (8μg/ml)
7	2-1 (8μg/ml)	35.8%	+	CRMA (8μg/ml)
8	2-1 (8μg/ml)	34.6%	+	CRMA (8μg/ml)
9	2-1 (8μg/ml)	37.5%	+	MuFADD-DN (8μg/ml)
10	2-1 (8μg/ml)	40.7%	+	MuFADD-DN (8μg/ml)
11	2-1 (8μg/ml)	14.9%	+	HuFADD-DN (8μg/ml)
12	2-1 (8μg/ml)	15.7%	+	HuFADD-DN (8μg/ml)
13	2-1 (8μg/ml)	<del>44.1%</del>	+	41.0% Hu-TRADD-DN (8μg/ml)
14	2-1 (8μg/ml)	<del>44.4%</del>	+	40.4% Hu-TRADD-DN (8μg/ml)
15	2-1 (8μg/ml)	<del>50.2%</del>	+	44.1% Hu-TRAF2-DN (8μg/ml)
16	2-1 (8μg/ml)	<del>50.2%</del>	+	43.7% Hu-TRAF2-DN (8μg/ml)
17	2-1 (8μg/ml)	<del>50.5%</del>	+	49.7% Hu-RIP-DN (8μg/ml)
18	2-1 (8μg/ml)	<del>52.7%</del>	+	52.7% Hu-RIP-DN (8μg/ml)
19	2-1 (8μg/ml)		+	Hu-RIP-DN (8μg/ml)

Add DEVD 4hrs after electroporation

pRK5 (16μg/ml)	17.4%	+DEVD	<del>25.0%</del>	20	50μM
pRK5 (8μg/ml)	2-1 (8μg/ml)	37.6%	+DEVD	21	50μM

Add ALLN (100ng/ml) 4 hrs before harvest

pRK5 (16μg/ml)		+ALLN	14.6%	21
pRK5 (8μg/ml)	2-1 (8μg/ml)	+ALLN	36.3%	23

Add cyclohexamide 4 hrs before harvest

pRK5 (16μg/ml)		+cyc	24.7%	24
pRK5 (8μg/ml)	2-1 (8μg/ml)	+cyc	46.6%	25

SAVED AS FACS

JS2

#26 1860/3 ~~51.7%~~ 43.9%

A - ROBERT

FACS

SAVED AS JS2

A - 3.6μg RIP WROTE

ADD 9μM SAT (QUINONE DIO DEVD)  
CONCENTRATION OF 0.5% (2:1)  
SAMPLES 6 CYC.

13 x 7μl = 91μl PE

12 x 7μl = 84μl ANA

13 x 0.4μl = 5.2μl

14 x 15μl = 210 ANA  
189:21μl

= 20, &amp; 21 WORKS SLOWLY UP. DOSES WERE WRONG ON 22 &amp; 25

#15 HAD VERY LOW #S OF CD4+ CELLS

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Recorded by

DNA LADDER FROM PG 84

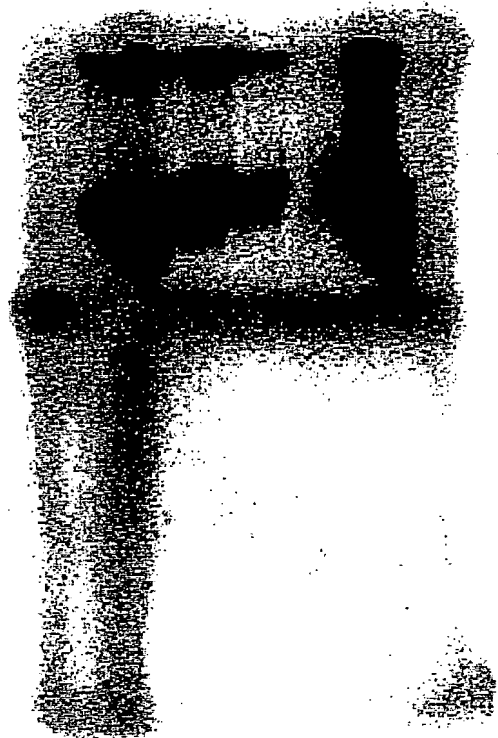
Project No. \_\_\_\_\_  
Book No. 26508

93

TITLE \_\_\_\_\_

From Page No. \_\_\_\_\_

#4 DAA



#4 DAA

DNA MUST  
BE

PREWARMED

50°C FOR 10 MIN

PRIOR TO LOADING!!

DNA + LOADING  
BUFFER 1ST WARMED  
TO 50°C BEFORE  
LOADING

GEL RUN @ 75 VOLTS 3HRS

DNA NOT WARMED TO 50°C  
GEL RUN 3HRS @ 100 VOLTS

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

Recorded by

NOTEBOOK NO. 27250

ISSUED TO JAMES SHORWEN

ON \_\_\_\_\_ 19 \_\_\_\_\_

DEPARTMENT MOLECULAR ONCOLOGY

**RETURNED**\_\_\_\_\_ **19**\_\_\_\_\_

—SCIENTIFIC NOTEBOOK CO.—

**2831 LAWRENCE AVE.**

**P.O. BOX 238**

**STEVENSVILLE, MI 49127**

**616-429-8285**

**ADE- 23**

USSN 10/052,798

TITLE

Project No. 27250  
Book No. 27250

Frc SAVO #1 317-97 cell  
IMAGOS AS. 293 cells (03/17/97)

1000K cells for DNA Ladder

1. <u>#1</u>	pRK5 (20µg/ml)	<u>0.9 µg/ml</u>
2. <u>1.1 µg/ml</u>	2-1 (10µg/ml)	+ pRK5 (10µg/ml)
3. <u>1.1</u>	2-1 (10µg/ml)	+ CRMA (10µg/ml)
4. <u>0.8</u>	2-1 (10µg/ml)	+ HuTRADD-DN (10µg/ml)
5. <u>0.9</u>	2-1 (10µg/ml)	+ HuTRAF2-DN (10µg/ml)
6. <u>0.7</u>	2-1 (10µg/ml)	+ HuFADD-DN (10µg/ml)
7. <u>2.1</u> <u>1.2</u>	2-1 (10µg/ml)	+ MuFADD-DN (10µg/ml)
8. <u>1.3 µg/ml</u>	2-1 (10µg/ml)	+ pRK5 (10µg/ml)
9.	pRKRMA (10µg/ml)	+ pRK5 (10µg/ml)
10. <u>1.0</u>	2-1 (10µg/ml)	+ RIP-DN (10µg/ml)

Add the DEVD-Fmk (200µM) when the medium is changed at 5hrs.

TRANSFECTED 11AM

GEL #1 LOADING

#1, #2, #3, #8

DEVD ALDHYD

GEL #2 LOADING

#4, #5, #6, #7, #10, <sup>DEVD-FMK</sup> #11

SAMPLE	VOLUME AFTER 1:5 DILUTION	H <sub>2</sub> O	10x KERNOW BUFFER	<sup>2</sup> sa DILUTION 1:20	KERNOW 672446
1	5.5	1.5	1 µl	1 µl	1 µl
2	4.5	2.5			
3	4.5	2.5			
4	6.25	0.75 <u>DEVD-FMK</u>			
5	5.6	1.4			
6	7.1				
7	<u>4.2</u>	<u>2.8</u>			
8	3.8	3.2			
9					
10	5	2			

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

Recorded by



## INCYTE construct 2-1

- Step 1 Electroporate 1,000K HeLa cells with 8μg of gene X, 8μg of gene pRK5,  
1μl VARNAs, and 4μg of pRKCD4.  
Step 2 Change medium after 4hrs.  
Step 3 Express for 24hrs and harvest for FACS.

A	(+) pRKCD4			PE
B	(-) pRKCD4	(+) pRK5		PE
C	(+) pRK5 + cdy		+Apo2	FITC + PE
D	(+) pRK5	-apoptosis		+ FITC

SAVOD PAS AS

- |   |       |               |   |                   |
|---|-------|---------------|---|-------------------|
| 1 | 13.0% | pRK5(16μg/ml) |   |                   |
| 2 | 43.1% | pRK5 (8μg/ml) |   | 2-1(8μg/ml)       |
| 3 |       | 2-1 (8μg/ml)  | + | CRMA(8μg/ml)      |
| 4 |       | 2-1 (8μg/ml)  | + | CRMA(8μg/ml)      |
| 5 | 30.4% | 2-1(8μg/ml)   | + | HuFADD-DN(8μg/ml) |
| 6 | 32.1% | 2-1(8μg/ml)   | + | HuFADD-DN(8μg/ml) |
| 7 | 41.9% | 2-1(8μg/ml)   | + | Hu-RIP-DN(8μg/ml) |

JS 2031897

Add DEVD 4hrs after electroporation (200μM)

- |   |       |                |             |                     |          |
|---|-------|----------------|-------------|---------------------|----------|
| 8 | 9.3%  | pRK5 (16μg/ml) |             | <del>DEVD</del> XVD | 4μl: 1ul |
| 9 | 14.4% | pRK5 (8μg/ml)  | 2-1(8μg/ml) | <del>DEVD</del> YVD | 4μl: 1ul |

10 15.0% pRK5 2-1 + ~~DEVD~~ DEVD 200μM 4μl: 1ul

11 10 x 0.4ul = 4.4ul PAS

11 10 x 1.5 = 15ul 18ul: 162ul ANOX17

11 10 x 0.7μl = 7.7μl

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

## INCYTE construct 2-1

- Step 1 Electroporate 1,000K HeLa cells with 8µg of gene X, 8µg of gene pRK5,  
1µl VARNAs, and 4µg of pRKCD4.  
Step 2 Change medium after 4hrs.  
Step 3 Express for 24hrs and harvest for FACS.

A	(+) pRKCD4			PE
B	(-) pRKCD4	(+) pRK5		PE
C	(+) pRK5		+Apo2	FITC + PE
D	(+) pRK5	-apoptosis		+ FITC
1	pRK5 (16µg/ml)			
2	pRK5 (8µg/ml)		2-1(8µg/ml)	
3	2-1 (8µg/ml)	+	CRMA(8µg/ml)	
4	2-1 (8µg/ml)	+	CRMA(8µg/ml)	
5	2-1(8µg/ml)	+	HuFADD-DN(8µg/ml)	
6	2-1(8µg/ml)	+	HuFADD-DN(8µg/ml)	

Add DEVD 4hrs after electroporation (200µM)

7	pRK5 (8µg/ml)	2-1(8µg/ml)	+DEVD
8	pRK5 (8µg/ml)	2-1(8µg/ml)	+DEVD

Add YVAD 4hrs after electroporation (200µM)

9	<sup>YVAD</sup> pRK5 (8µg/ml)	2-1(8µg/ml)	+YVAD
10	pRK5 (8µg/ml)	2-1(8µg/ml)	+YVAD

$$RBS = 11 \times 0.425 = 4.675$$

PF 25µl 77µl

Amoxin 1600µg/100.5

180:162

BACKGROUND APOPTOSIS WAS TOO HIGH. I USED  
UNHEALTHY HOST CELLS IN THE TRANSFECTION.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

From Page No. \_\_\_\_\_

1 CHLOROFORM EXTRACT, REPRECIPITATION, 70% ETCH WASH, &amp; SUSPENSION

1 mg FIBER  
1:5 DILUTION

1:4 DILUTION

1:3 DILUTION

H<sub>2</sub>O

1	0.73	mg / $\mu$ l	6.8 $\mu$ l	5.5 $\mu$ l		1.5 $\mu$ l
2	0.89		5.6 $\mu$ l	4.5 $\mu$ l		2.5 $\mu$ l
3	0.80		6.3 $\mu$ l	6.2 $\mu$ l		0.8 $\mu$ l
4	0.63	<del>0.80</del>			4.8 $\mu$ l	2.2 $\mu$ l
5	0.64				4.7 $\mu$ l	2.3 $\mu$ l
6	0.59				5.1 $\mu$ l	1.9 $\mu$ l
7	1.04			3.8 $\mu$ l		3.2 $\mu$ l
8	0.80			5 $\mu$ l		2 $\mu$ l
10	0.83			4.8 $\mu$ l		2.2 $\mu$ l
A	0.70			5.7 $\mu$ l		1.3 $\mu$ l

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Recorded by

HeLa (03-21-97)

HeLa (plated in HGD MEM)

Dose response in the presence and absence of ALLN (inhibitor of I $\kappa$ B degradation) or cycloheximide. Plate ~200K cells in 6 well plates on the previous night. Cells will be pretreated with ALLN or cycloheximide for 1 hr prior to Apo2L. Cells will be incubated with Apo2L for 5hrs prior to annexin staining.

100 PCS AS:

JSI 032297

9:20AM pretreat 12 wells +/- ALLN @ 40 $\mu$ g/ml (1:1000) for 1hr.

10:20AM treat cells with each ligand @ 1 $\mu$ g/ml.

3:20PM Harvest and annexin stain (no PI).

Apo2L		TNF		TNF	
1 6.4% c/c.	+ NT	13 14.7% c/c.	+ NT	25 ALLN + NT	15.6%
2 8.5% c/c.	+1:500	14 14.5% c/c.	+1:500	26 ALLN +1:500	59.0%
3 12.4% c/c.	+1:1000	15 17.5% c/c.	+1:1000	27 39.7% ALLN +1:1000	<del>59.0%</del>
4 14.7% c/c.	+1:2000	16 16.1% c/c.	+1:2000	28 35.2% ALLN +1:2000	<del>59.0%</del>
5 14.6% c/c.	+1:4000	17 11.1% c/c.	+1:4000	29 36.7% ALLN +1:4000	<del>59.0%</del>
6 14.6% c/c.	+1:8000	18 65.5% c/c.	+1:8000	30 32.0% ALLN +1:8000	<del>59.0%</del>

0.5 $\mu$ l = 19.6 PBS

1.5 $\mu$ l = 57.1 ANNEXIN

7% 513 $\mu$ l

(2.5X ALLN)

7 7.3%	NT	19 8.4%	NT	31 ALLN + NT	14.0%
8 72.3%	1:500	20 15.0%	1:500	32 ALLN +1:500	43.0%
9 63.5%	1:1000	21 15.6%	1:1000	33 ALLN +1:1000	32.7%
10 41.1%	1:2000	22 10.9%	1:2000	34 ALLN +1:2000	27.6%
11 24.5%	1:4000	23 10.9%	1:4000	35 ALLN +1:4000	21.3%
12 10.0%	1:8000	24 10.5%	1:8000	36 ALLN +1:8000	21.6%

MINIMUM OF 30,000 GATED EVNTS

To Page No. \_\_\_\_\_

Witnessed & Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

From \_\_\_\_\_

SAVED FACS AS%

JS2032497

## INCYTE construct 2-1

- Step 1 Electroportate 1,000K HeLa cells with 8μg of gene X, 8μg of gene pRK5, 1μl VARNAs, and 4μg of pRKCD4.  
Step 2 Change medium after 4hrs.  
Step 3 Express for 24hrs and harvest for FACS.

A	(+) pRKCD4	(+) pRK5	PE
B	(-) pRKCD4		PE
C	(+) pRK5 + pRKCD4	+Apo2	FITC + PE
D	(+) pRK5	-apoptosis	+ FITC

$$7\mu\text{l} \times 13 \times = 91\mu\text{l}$$

$$1.5\mu\text{l} \times 13 = 19.5\% \text{ } 175.5$$

$$0.4 \times 13 = 5.2\% \text{ } \text{PBS}$$

1	pRK5(16μg/ml)	11.6%	
2	pRK5 (8μg/ml)	29.2%	2-1(8μg/ml)
3	2-1 (8μg/ml)	11.3%	+
4	2-1 (8μg/ml)	16.7%	+
5	2-1(8μg/ml)	20.3%	+
6	2-1(8μg/ml)	22.4%	+
7	2-1(8μg/ml)	38.5%	+

CRMA(8μg/ml)  
CRMA(8μg/ml)

HuFADD-DN(8μg/ml)  
HuFADD-DN(8μg/ml)

Hu-RIP-DN(8μg/ml)

Add DEVD 4hrs after electroporation (200μM)

8	pRK5 (8μg/ml)	17.2%	2-1(8μg/ml)	+DEVD
9	pRK5 (8μg/ml)	19.0%	2-1(8μg/ml)	+DEVD

Add YVAD 4hrs after electroporation (200μM)

10	pRK5 (8μg/ml)	11.5%	2-1(8μg/ml)	+YVAD
11	pRK5 (8μg/ml)	9.1%	2-1(8μg/ml)	+YVAD

12 pRK5 9.4%

SECOND #10 IS #11

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

From Page No. \_\_\_\_\_

## INCYTE construct 2-1

- Step 1 Electroporate 1,000K HeLa cells with 8µg of gene X, 8µg of gene pRK5,  
1µl VARNA, and 4µg of pRKCD4.  
Step 2 Change medium after 4hrs.  
Step 3 Express for 24hrs and harvest for FACS.

A	(+) pRKCD4		PE
B	(-) pRKCD4	(+) pRK5	PE
C	(+) pRK5 + pRKCD4	+Apo2	FITC + PE
D	(+) pRK5	-apoptosis	+ FITC

1	7.9%	pRK5 (16µg/ml)	
2	31.9%	pRK5 (8µg/ml)	2-1 (8µg/ml)
3	33.4%	pRK5 (8µg/ml)	2-1 (8µg/ml)
4	22.0%	2-1 (8µg/ml)	+ HuFADD-DN (8µg/ml)
5	25.7%	2-1 (8µg/ml)	+ HuFADD-DN (8µg/ml)
6	26.7%	2-1 (8µg/ml)	+ HuFADD-DN (8µg/ml)
7	42.9%	2-1 (8µg/ml)	+ Hu-RIP-DN (8µg/ml)
8	40.9%	2-1 (8µg/ml)	+ Hu-RIP-DN (8µg/ml)

SAVED FACS AS:  
JS 2032797C

$$9 \times 0.4 = 3.6$$

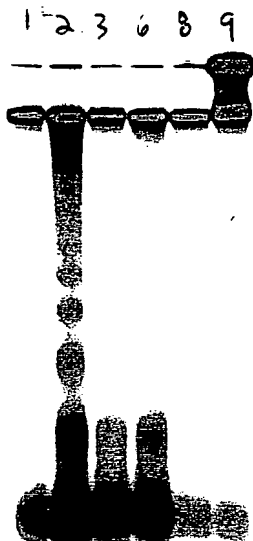
$$9 \times 15 = 135$$

1215.13.5 AMOXIN

$$9 \times 7 \mu l = 63 \mu l PO$$

$$10 \times 15 = 135.15 \mu l AMOXIN$$

SAVED AS JS 3-22-97 35h



Notes on Trevigen DNA Ladder Kits

**Additional extraction-** you must include a phenol/chloroform extraction followed by a chloroform extraction between steps 7 and 8.

**Thick Agarose Gel-** you must use at least 75mls in pouring your mini agarose gel (0.9cm).

**Warm the Samples-** you must warm the labeled DNA containing loading dye to 50°C for 5 min prior to loading on the gel.

-use 1µg of DNA in your reactions.

-use 1.5% Trevicil agarose.

-run 3hrs at 75Volts (cut off the gel below the bromophenol blue).

-do not fix the gel, rinse 5min in TAE buffer and dry 2hrs at 60°C.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

293 cells 03/24/97

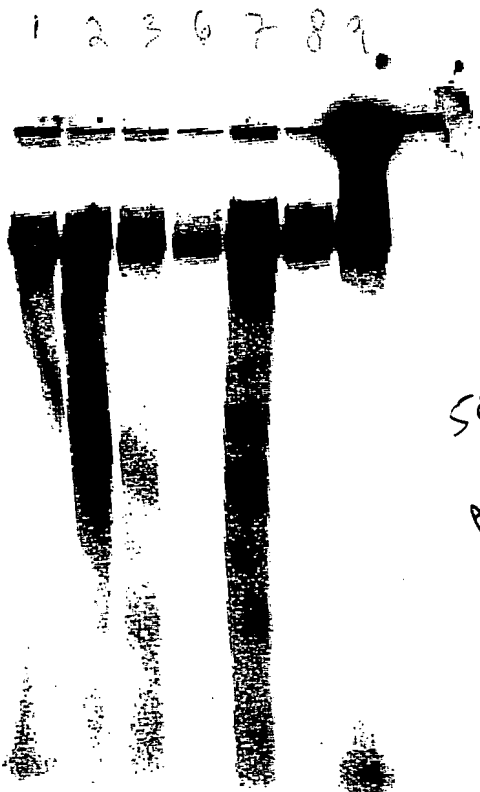
1000K cells for DNA Ladder

- |     |                |   |                           |
|-----|----------------|---|---------------------------|
| 1.  | pRK5 (20µg/ml) |   |                           |
| 2.  | 2-1 (10µg/ml)  | + | pRK5 (10µg/ml)            |
| 3.  | 2-1 (10µg/ml)  | + | CRMA (10µg/ml)            |
| 4.  | 2-1 (10µg/ml)  | + | HuTRADD-DN (10µg/ml)      |
| 5.  | 2-1 (10µg/ml)  | + | HuTRAF2-DN (10µg/ml)      |
| 6.  | 2-1 (10µg/ml)  | + | HuFADD-DN (10µg/ml)       |
| 7.  | 2-1 (10µg/ml)  | + | MuFADD-DN (10µg/ml)       |
| 8.  | 2-1 (10µg/ml)  | + | pRK5 (10µg/ml) + DEVD-Fmk |
| 9.  | 2-1 (10µg/ml)  | + | pRK5 (10µg/ml) + YVAD-Fmk |
| 10. | 2-1 (10µg/ml)  | + | RIP-DN (10µg/ml)          |

DNA concentration	1µg APT200 DILUTION 1:3	H <sub>2</sub> O
0.85	3.5 µl	3.5 µl
0.50	6 µl	1 µl
0.44	6.8 µl	0.2 µl
0.64	4.7 µl	2.3 µl
0.50	6 µl	1 µl
0.53	5.7 µl	1.3 µl
0.79	3.8 µl	3.2 µl
0.51	5.9 µl	1.1 µl
0.72	4.2 µl	2.8 µl
0.56	5.4 µl	1.6 µl

Add the DEVD-Fmk (200µM) at 4 min. DHN.

1 hr 20 min exposure

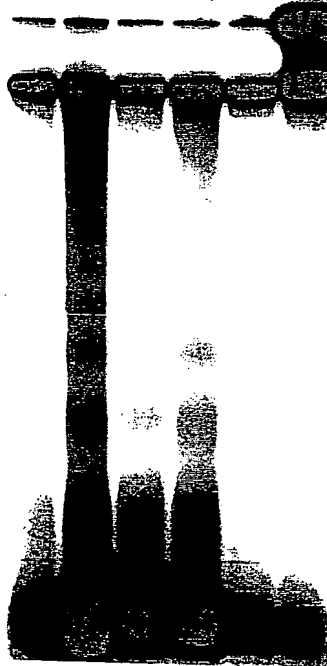


- REPEAT USING 100 mlb OF AGAROSE MIX

- REPEAT, SAME

15 3-27-97

#1 #2 #3 #4 #5 #6

SOME OF AGAROSE  
SAME DNA BUT 100 mlb OF AGAROSE

Witnessed &amp; Understood by me,

Date

Invented by

a8

Recorded by

No.

From Page No. \_\_\_\_\_

USING DATA FROM SS 4 PG 85 &amp; 95 &amp; SS 5 PG 7

APO2L (-ALLN - CYCLOHEXIMIDE)

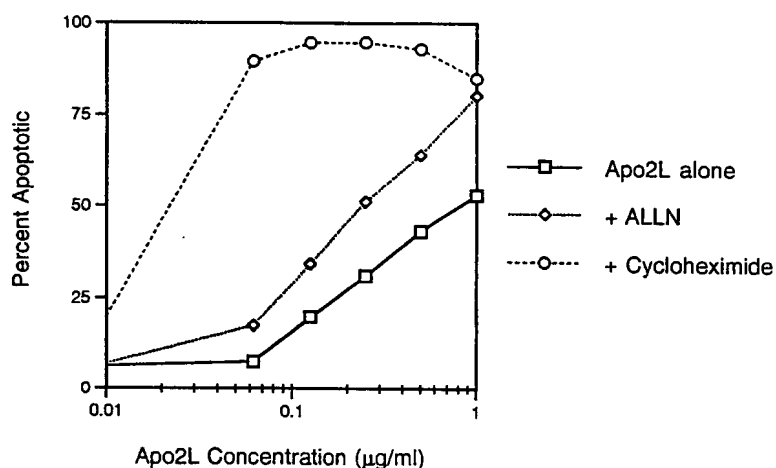
0 6%  
 0.0625 7.4%  
 0.125 19.7%  
 0.250 31.1%  
 0.5 43.2%  
 1.0 53.0%

Data 03-25-97

Tue, Mar 25, 1997 5:14 PM Page 1

	1	2	3	4	5
	Apo2L Concentral	Apo2L alone	Column 3	+ ALLN	- Cycloheximide
1	0	0.06		0.048	0.064
2	0.0625	0.074		0.174	0.896
3	0.125	0.197		0.344	0.946
4	0.25	0.311		0.512	0.947
5	0.5	0.432		0.64	0.929
6	1	0.53		0.803	0.85

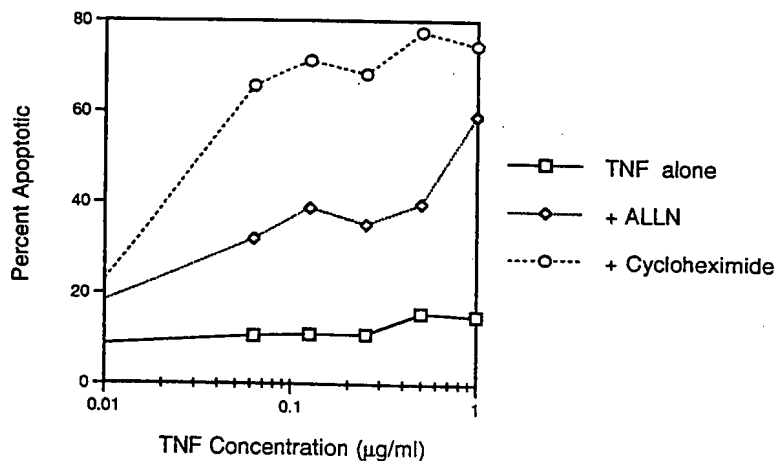
HeLa Apo2L Dose Response



TNF (-ALLN - CYCLOHEXIMIDE)

0 7.4%  
 0.0625 8.1%  
 0.125 8.4%  
 0.250 8.4%  
 0.5 9.7%  
 1.0 10.9%

HeLa TNF Dose Response



To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Recorded by



From Page No. \_\_\_\_\_

- Step 1 Electroportate 1,000K HeLa cells with 8μg of gene X, 8μg of gene pRK5,  
1μl VARNA, and 4μg of pRKCD4.  
Step 2 Change medium after 4hrs.  
Step 3 Express for 24hrs and harvest for FACS.

A	(+) pRKCD4			PE
B	(-) pRKCD4	(+) pRK5		PE
C	(+) pRK5 + pRKCD4	+Apo2	FITC + PE	
D	(+) pRK5	-apoptosis	+ FITC	
19.7%	1	2-1(4μg/ml)	HuFADD-DN(12μg/ml)	+ pRK5(0μg/ml)
21.0%	2	2-1(4μg/ml)	HuFADD-DN(8μg/ml)	+ pRK5(4μg/ml)
30.6%	3	2-1(4μg/ml)	HuFADD-DN(4μg/ml)	+ pRK5(8μg/ml)
29.8%	4	2-1(4μg/ml)	HuFADD-DN(0μg/ml)	+ pRK5(12μg/ml)
10.5%	5	pRK5(4μg/ml)	HuFADD-DN(12μg/ml)	+ pRK5(0μg/ml)
10.4%	6	pRK5(4μg/ml)	HuFADD-DN(8μg/ml)	+ pRK5(4μg/ml)
11.4%	7	pRK5(4μg/ml)	HuFADD-DN(4μg/ml)	+ pRK5(8μg/ml)
13.2%	8	pRK5(4μg/ml)	HuFADD-DN(0μg/ml)	+ pRK5(12μg/ml)

$$10 \times 15 \mu\text{L ANNOXIN} =$$

$$13.5 \times 121.5$$

SAVED FACS 15%

$$10 \times 0.4 \mu\text{L} =$$

$$4.25$$

152040397

$$9 \times 0.7 \mu\text{L} =$$

$$45 \mu\text{L PO}$$

10,000 GATT

ORONIS

To Page No. \_\_\_\_\_

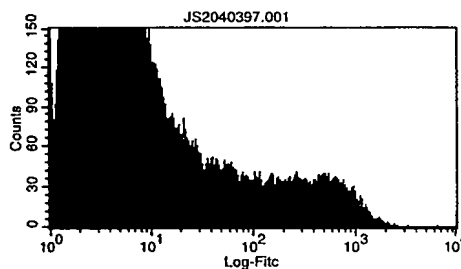
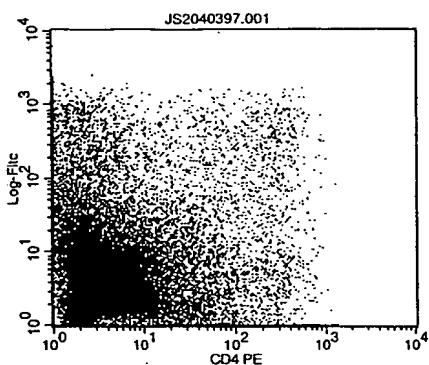
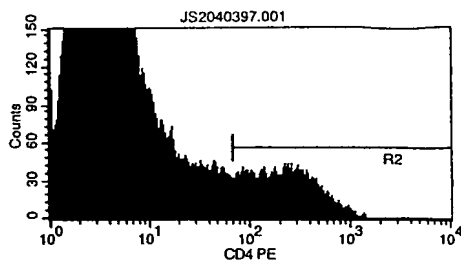
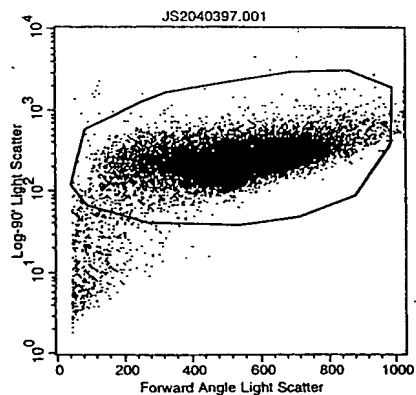
Witnessed &amp; Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by \_\_\_\_\_

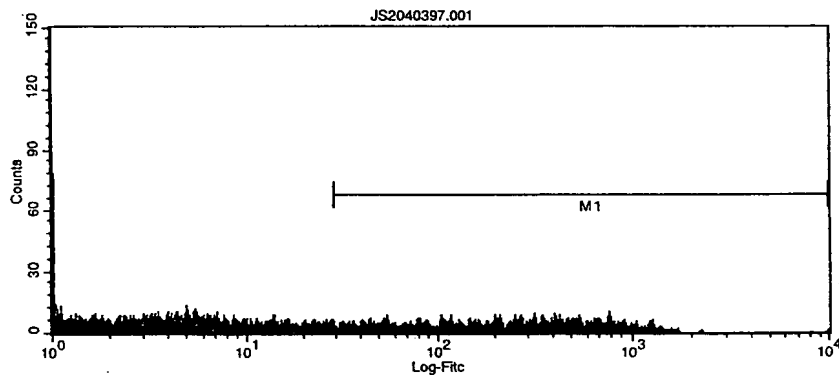
Date \_\_\_\_\_

Recorded by \_\_\_\_\_



Sample ID: 1 Gate: G3  
Gated Events: 7136 Total Events: 104304

Marker	Events	% Gated	% Total
All	7136	100.00	6.84
M1	1406	19.70	1.35



To Page No. \_\_\_\_\_

Witnessed & Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

From Page No. \_\_\_\_\_

## INCYTE construct 2-1

- Step 1 Electroportate 1,000K HeLa cells with 8µg of gene X, 8µg of gene pRK5,  
1µl VARNA, and 4µg of pRKCD4.  
Step 2 Change medium after 4hrs.  
Step 3 Express for 24hrs and harvest for FACS.

A	(+) pRKCD4		PE
B	(-) pRKCD4	(+) pRK5	PE
C	(+) pRK5 + pRKCD4	+Apo2	FITC + PE
D	(+) pRK5	-apoptosis	+ FITC

1	11.5%	pRK5 (16µg/ml)		
2	20.6%	2-1 (8µg/ml)	+	HuFADD-DN (8µg/ml)
3	20.4%	2-1 (8µg/ml)	+	HuFADD-DN (8µg/ml)
4	24.2%	2-1 (8µg/ml)	+	HuFADD-DN (8µg/ml)
5	24.7%	2-1 (8µg/ml)	+	HuFADD-DN (8µg/ml)
6	25.3%	2-1 (8µg/ml)	+	HuFADD-DN (8µg/ml)
7	36.5%	2-1 (8µg/ml)	+	pRK5 (8µg/ml)
8	38.4%	2-1 (8µg/ml)	+	pRK5 (8µg/ml)
9	41.7%	2-1 (8µg/ml)	+	pRK5 (8µg/ml)
10	36.0%	2-1 (8µg/ml)	+	pRK5 (8µg/ml)
11	36.3%	2-1 (8µg/ml)	+	pRK5 (8µg/ml)

electroporation/purified @ 2PM

24 HRS CULTURE

CHANGE MEDIUM @ 5PM

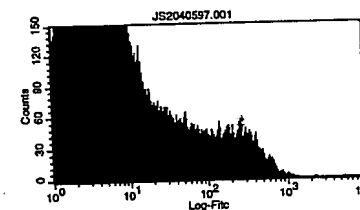
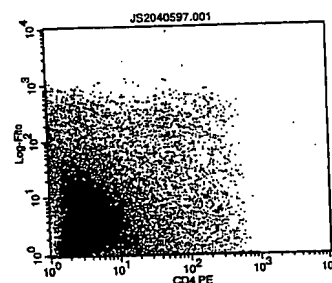
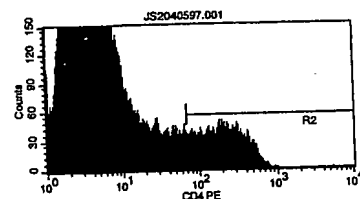
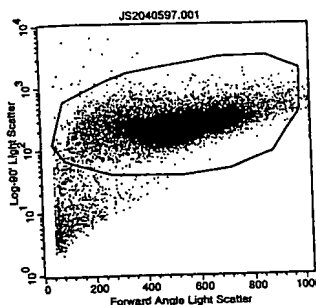
FACS SAVED AS:

JS2040597

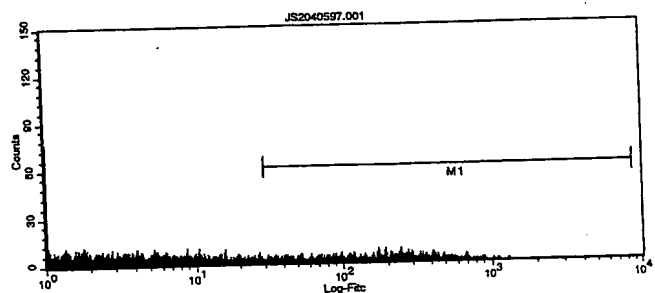
$$12 \times 0.4 \mu\text{l} = 4.8 \mu\text{l}$$

$$13 \times 15 \mu\text{l} \text{ ANOXON} = 19.5 \mu\text{l} \text{ @ } 175.5 \mu\text{l}$$

$$7 \mu\text{l} \times 12 = 84 \mu\text{l} \text{ PO}$$



Sample ID: 1	Gate: G3		
Gated Events: 7142	Total Events: 117008		
Marker	Events	% Gated	% Total
All	7142	100.00	6.10
M1	821	11.50	0.70



Witnessed &amp; Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented \_\_\_\_\_

Recorded by \_\_\_\_\_

Pag \_\_\_\_\_

# SUMMARY OF HOUT TRANSPORTATION DATA

Project No. \_\_\_\_\_  
Book No. **27250**

25

From Page No. \_\_\_\_\_

PRK5

PRK5+2-1

HuFADD-DN+2-1

~~Z-VAD~~  
~~X-VAD+2-1~~

9.4  
11.6  
8.6  
9.7  
14.1  
13  
11.5

29.8  
28.6  
43.9  
35.4  
43.1  
29.2  
31.9  
33.4  
36.5  
38.4  
41.7  
36.0  
36.3

22.4  
20.3  
14.9  
15.7  
28.9  
30.4  
32.1  
22.0  
25.7  
26.7  
20.6  
20.4  
24.2  
24.7  
25.3

9.3  
11.5  
9.1  
10.0 ± 1.3

DEVD+2-1

14.4  
17.2  
19.0  
16.9 ± 2.3

11.1 % ± 2.0

35.7 ± 5.1

23.6 ± 4.9

MuFADD-DN+2-1

HuTRADD-DN+2-1

HuTRAF2-DN+2-1

37.1  
40.7  
48.5

42.2 ± 5.6

41.0  
40.0  
42.5

41.2 ± 1.2

50.8  
44.1  
43.7

46.2 ± 4.0

PRKCRMA+2-1

HuRIP-DN+2-1

11.4  
16.7  
11.3

13.1 ± 3.1

49.7  
52.7  
41.9  
38.5  
42.9  
40.9

44.4 ± 5.5

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

Recorded by

*Jan Smith*

4-6-97

04-23-97

Plated HeLa cells (10mMEDTA) @ 100K cells/well in 12 well dishes previous evening.

10:30

11AM pre-incubate the Apo2L (make a 1µg/ml stock using 3.3 µl in 3 ml) +/- ECD (100nM) and +/- FLAG Mab (1µg/µl-dilute to 1µg/µl by adding 5µl to 10µl medium) for 1hr.

11:30 → 4:30

Pre-incubate the cells in cycloheximide 1 hr at 50µg/ml.

## I NO CYCLOHEXIMIDE

1 64.9% 0.5 µg/ml	Apo2L +	Tris Buffer (50µl)	+ Anti-Flag Mab (1µg/ml)
2 49.3 0.3 µg/ml	Apo2L +	Tris Buffer (50µl)	+ Anti-Flag Mab (1µg/ml)
3 12.5 NT	+	Tris Buffer (50µl)	+ Anti-Flag Mab (1µg/ml)
4 13.4 0.5 µg/ml	Apo2L +	ECD (50µl)	+ Anti-Flag Mab (1µg/ml)
5 11.0 0.3 µg/ml	Apo2L +	ECD (50µl)	+ Anti-Flag Mab (1µg/ml)
6 11.4 NT	+	ECD (50µl)	+ Anti-Flag Mab (1µg/ml)

## II

Preincubate and include cycloheximide

7 21.4% 0.03 µg/ml	Apo2L +	Tris Buffer (50µl)	+ Anti-Flag Mab (1µg/ml)
8 15.5 0.01 µg/ml	Apo2L +	Tris Buffer (50µl)	+ Anti-Flag Mab (1µg/ml)
9 15.0 NT	+	Tris Buffer (50µl)	+ Anti-Flag Mab (1µg/ml)
10 13.7 0.03 µg/ml	Apo2L +	ECD (50µl)	+ Anti-Flag Mab (1µg/ml)
11 13.9 0.01 µg/ml	Apo2L +	ECD (50µl)	+ Anti-Flag Mab (1µg/ml)
12 14.7 NT	+	ECD (50µl)	+ Anti-Flag Mab (1µg/ml)

Kodak Anti-Flag M2 Mab 3µg/µl stock.

#12 4-24-97



#42 4-24-97



THE APO4 PURIFIED  
ECD FROM BOB  
PITTI BLOCKED  
APOPTOSIS INDUCED  
BY APO2 LIGAND

USG #16 4-24-97  
AS THE FIGURE  
2 TAKEN AFTER  
3 HRS

SAVED FIGURE ON JAMIE MICROSCOPE IMAGES

Date

4-24-97

Invented by

Recorded by

Date

MAKE 3.3

OF 1.1 µg/µl APO2L STOCK 6.6 µl = 3.3

DILUTE 1:10 AND  
ADD CYCLOHEXIMIDE

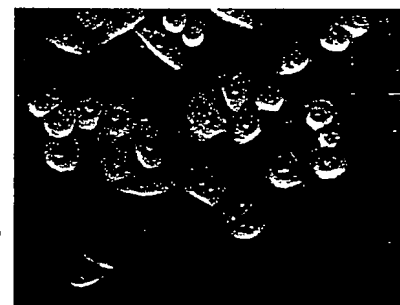
SAVED FILES AS:

252042497

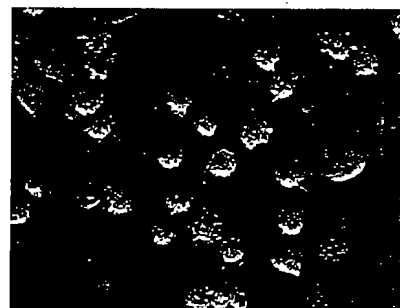
SAVED COLLECTION 40,000

EVENTS

#4  
4-24-97  
SMA



#1  
4-24-97  
SMA



SAVED ON JAMIE MICROSCOPE IMAGES

#2

MICROSCOPE IMAGES 2  
10 Page No.

From Page No.

(03-24-97)

HeLa (plated in HGD MEM)

Dose response in the presence and absence of ALLN (inhibitor of I $\kappa$ B degradation) or cycloheximide. Plate ~200K cells in 6 well plates on the previous night. Cells will be pretreated with ALLN or cycloheximide for 1 hr prior to Apo2L. Cells will be incubated with Apo2L for 5hrs prior to annexin staining.

8:00AM pretreat 12 wells +/- ALLN @ 40 $\mu$ g/ml (1:1000) for 1hr.9:00AM treat cells with each ligand @ 1 $\mu$ g/ml.

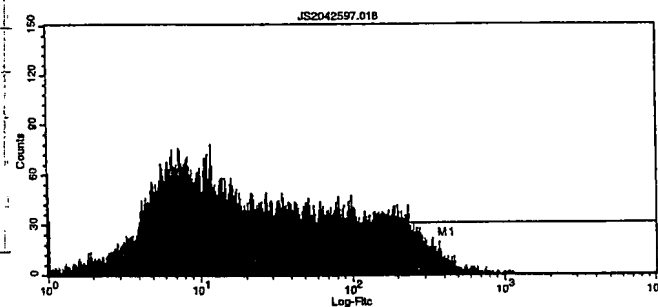
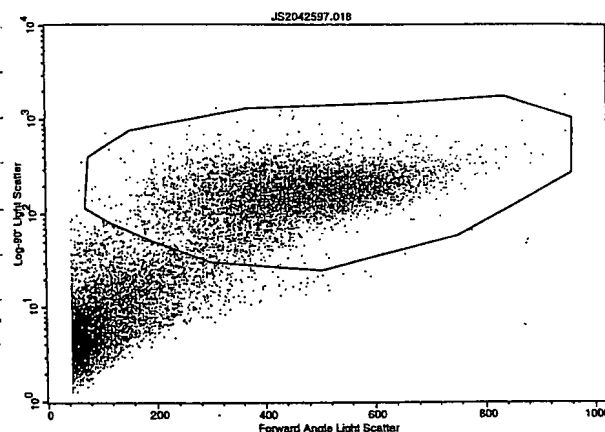
2:00PM Harvest and annexin stain (no PI).

1	NT	5.6%	
2	NT	5.1%	
3	NT	5.7%	8.0%
4	ALLN + NT		7.0%
5	ALLN + NT		7.4%
6	ALLN + NT		
7	cyc. + NT	7.0%	
8	cyc. + NT	6.3%	
9	cyc. + NT	8.9%	
10	Apo2L 1 $\mu$ g/ml	25.5%	
11	Apo2L 1 $\mu$ g/ml	29.0%	
12	Apo2L 1 $\mu$ g/ml	30.6%	
13	cyc. + Apo2L 1 $\mu$ g/ml	51.9%	
14	cyc. + Apo2L 1 $\mu$ g/ml	58.9%	
15	cyc. + Apo2L 1 $\mu$ g/ml	61.8%	
16	ALLN + Apo2L 1 $\mu$ g/ml	58.2%	
17	ALLN + Apo2L 1 $\mu$ g/ml	59.2%	
18	ALLN + Apo2L 1 $\mu$ g/ml	61.3%	

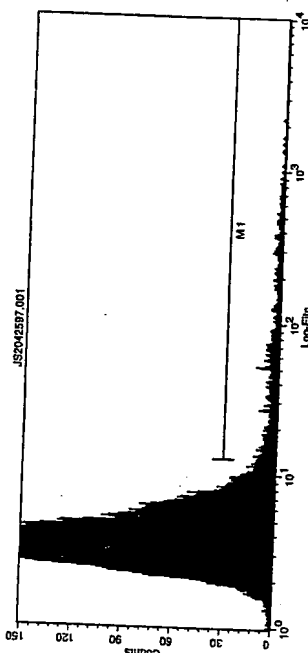
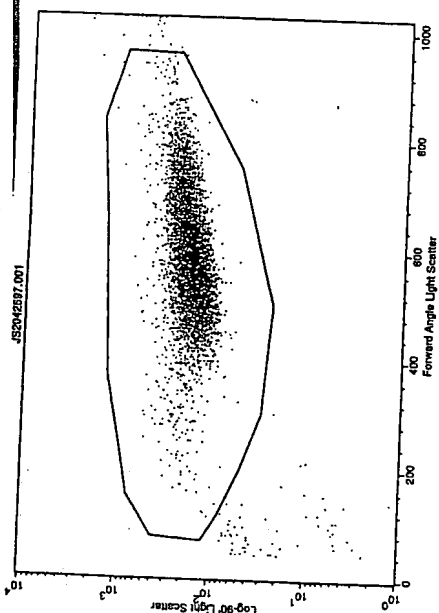
SAVED FACS AS

JS2042597 1-18

COLLECTED 20,000 G1 EVENTS



Sample ID: 18 Gated Events: 20203  
Total Events: 45840  
Marker % Gated  
All 100.00  
M1 61.29



Gated Events: 20196

Sample ID: 1  
Total Events: 20405  
Marker % Gated  
All 100.00  
M1 6.85

THESE PLOTS WERE  
REALLY NOISY AND EACH  
INDIVIDUAL CELL MAY HAVE  
SEEN LESS DRUG THAN  
USUAL (MEANING LESS APO2L)

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Recorded by

04-29-97 FC IC50

Plated HeLa cells (10mMEDTA) @ 250Kcells/well in 12 well dishes previous evening. Preincubate the Apo2L (0.55μl/0.5ml) +/-Apo2 Fc for 1hr.

1:30 → 6:30

- 1 A Apo2L (1μg/ml) + Fc (27μg/ml)  
 2 B Apo2L (1μg/ml) + Fc (9μg/ml)  
 3 C Apo2L (1μg/ml) + Fc (3μg/ml)  
 4 D Apo2L (1μg/ml) + Fc (1μg/ml)  
 5 E Apo2L (1μg/ml) + Fc (.33μg/ml)  
 6 G Apo2L (1μg/ml) + Fc (.11μg/ml)  
 7 H Apo2L (1μg/ml) + Fc (.037μg/ml)  
 8 J Apo2L (1μg/ml) + Fc (.012μg/ml)  
 9 K Apo2L (1μg/ml) + Fc (.004μg/ml)  
 10 L Apo2L (1μg/ml) + Fc (.0014μg/ml)  
 11 M Apo2L (1μg/ml) + Fc (.0004μg/ml)  
 12 N Apo2L (1μg/ml) + medium

10 μl STRAIGHT  
33 μl

5 μl + 10 μl MEDIUM ⇒ USE 10 μl ADD 10 μl cell...

A 10.8%  
 B 10.1%  
 C 9.3%  
 D 11.4%  
 E 12.8%  
 G 15.0%  
 H 31.9%  
 J 47.4%  
 K 62.0%  
 L 60.6%  
 M 56.1%  
 N 64.1%

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Recorded by

04-29-97

Plated HeLa cells (10mMEDTA) @ 150K, 200K, 250K, and 300K cells/well in 12 well dishes previous evening. The size of Apo2 is ~44Kd and the Fc should be about 100Kd, therefore use a 1:4 mass to mass ratio.

9AM pre-incubate the Apo2L (make a 1µg/ml stock using 3.3 µl in 3 ml) +/- IgG/Fc (2µg/ml) for 1hr. Make a 0.25 µg/µl stock of Apo2 Fc by diluting in medium and use 5µl/well.

0 NT 8.2%  $13 \times 0.5 = 6.5$   
1 Apo2L (0.5 µg/ml) 57.5%  $7.2 \mu\text{l} = 6.5$   
2 Apo2L (0.5 µg/ml) 54.2%  
3 Apo2L (0.5 µg/ml) 57.4%

I. (20 µg/ml) Apo2L Fc (stock is 2.7 mg/ml)

4 Apo2L (0.5 µg/ml) + Apo2 Fc (3.7µl) 8.5%  
5 Apo2L (0.5 µg/ml) + Apo2 Fc (3.7µl) 10.3%  
6 Apo2L (0.5 µg/ml) + Apo2 Fc (3.7µl) 10.6%  
7 NT + Apo2 Fc (3.7µl) 9.4%  
8 NT + Apo2 Fc (3.7µl) 10.4%  
9 NT + Apo2 Fc (3.7µl) 8.3%

II. 20 µg/ml TNF IgG (dilute 1:10 Scot's 20 µg/µl stock in medium)

10 Apo2L (0.5 µg/ml) + TNFR1 IgG (5µl) 62.6%  
11 Apo2L (0.5 µg/ml) + TNFR1 IgG (5µl) 62.9%  
12 Apo2L (0.5 µg/ml) + TNFR1 IgG (5µl) 65.3%  
13 NT + TNFR1 IgG (5µl) 10.8%  
14 NT + TNFR1 IgG (5µl) 10.0%  
15 NT + TNFR1 IgG (5µl) 10.1%

III. DR4 Fc

16 Apo2L (0.5 µg/ml) + DR4 Fc (50µl) 25 17.1%  
17 Apo2L (0.5 µg/ml) + DR4 Fc (50µl) 25 17.2%  
18 Apo2L (0.5 µg/ml) + DR4 Fc (50µl) 25 16.9%  
19 NT + DR4 Fc (50µl) 25 8.8%  
20 NT + DR4 Fc (50µl) 25 10.5%  
21 NT + DR4 Fc (50µl) 25 8.9%

THE Apo2Fc BLOCKS  
APO2L INDUCED APOPTOSIS

SAVED MICROSCOPE IMAGES ON MICROSCOPE IMAGES #2

#2 4-30-97

#6

#12

#18

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

4-30-97

Invented by

Date

Recorded by

JAMES SPENCER



HeLa (plated in HGD MEM)

Dose response in the presence and absence of ALLN (inhibitor of I $\kappa$ B degradation) or cycloheximide. Plate ~200K cells in 6 well plates on the previous night. Cells will be pretreated with ALLN or cycloheximide for 1 hr prior to Apo2L. Cells will be incubated with Apo2L for 5hrs prior to annexin staining.

9:20

8:00AM pretreat 12 wells +/- ALLN @ 40 $\mu$ g/ml (1:1000) for 1hr.

10:20

9:00AM treat cells with each ligand @ 0.5 $\mu$ g/ml and 0.25 $\mu$ g/ml

3:20

2:00PM Harvest and annexin stain (no PI).

1	NT	6.7%
2	NT	5.9%
3	NT	5.4%
4	ALLN + NT	6.5%
5	ALLN + NT	7.3%
6	ALLN + NT	7.8%
7	cyc. + NT	10.7%
8	cyc. + NT	8.5%
9	cyc. + NT	9.1%
10	Apo2L $\mu$ g/ml	26.7%
11	Apo2L $\mu$ g/ml	29.4%
12	Apo2L $\mu$ g/ml	29.9%

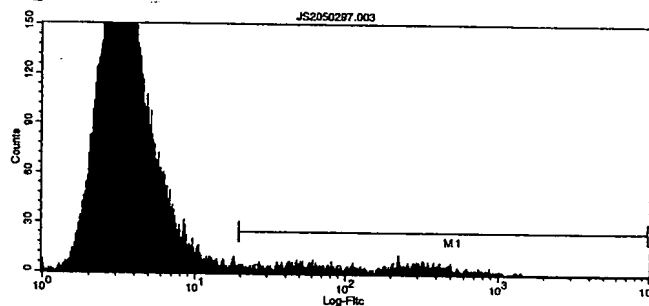
13	cyc. + Apo2L $\mu$ g/ml	73.2%
14	cyc. + Apo2L $\mu$ g/ml	67.2%
15	cyc. + Apo2L $\mu$ g/ml	75.3%

16	ALLN + Apo2L $\mu$ g/ml	52.2%
17	ALLN + Apo2L $\mu$ g/ml	48.7%
18	ALLN + Apo2L $\mu$ g/ml	55.8%

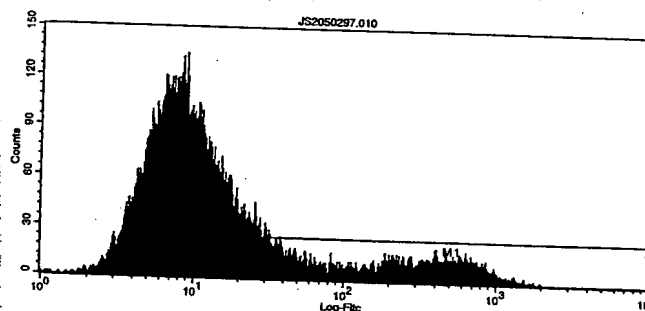
11  $\mu$ l: 10  $\mu$ l  
0.5 = 7.7  $\mu$ l: 7  $\mu$ l  
0.25 = 3.8  $\mu$ l: 7  $\mu$ l  
5.5  $\mu$ l: 10  $\mu$ l

7.6  
6.9  $\mu$ l: 5.7  $\mu$ l

19:17  $\mu$ l



Sample ID: 3  
Total Events: 20207  
Gated Events: 20073  
Marker % Gated  
All 100.00  
M1 5.44



Sample ID: 10  
Total Events: 22626  
Gated Events: 20183  
Marker % Gated  
All 100.00  
M1 26.19

To Page No.

Witnessed &amp; Understood by me,

Date

Invented by

Date

5-5-97

Recorded by

AMSS STOREMAN

From Page No. \_\_\_\_\_

04-29-97 FC IC50

SAURO FACS AS:

JS2050997

Plated HeLa cells (10mMEDTA) @ 250Kcells/well in 12 well dishes previous evening. Preincubate the Apo2L (1.1μl/0.1ml) +/- Apo2 Fc for 1hr.

## I. Apo2 Fc

- 1 Apo2L (1μg/ml) + Fc (27μg/ml)  
 2 Apo2L (1μg/ml) + Fc (9μg/ml)  
 3 Apo2L (1μg/ml) + Fc (3μg/ml)  
 4 Apo2L (1μg/ml) + Fc (1μg/ml)  
 5 Apo2L (1μg/ml) + Fc (.33μg/ml)  
 6 Apo2L (1μg/ml) + Fc (.11μg/ml)  
 7 Apo2L (1μg/ml) + Fc (.037μg/ml)  
 8 Apo2L (1μg/ml) + Fc (.012μg/ml)  
 9 Apo2L (1μg/ml) + Fc (.004μg/ml)  
 10 Apo2L (1μg/ml) + Fc (.0014μg/ml)  
 11 Apo2L (1μg/ml) + Fc (.0004μg/ml)  
 12 Apo2L (1μg/ml) + medium

## II. DR4 Fc

- 13 Apo2L (1μg/ml) + Fc (27μg/ml)  
 14 Apo2L (1μg/ml) + Fc (9μg/ml)  
 15 Apo2L (1μg/ml) + Fc (3μg/ml)  
 16 Apo2L (1μg/ml) + Fc (1μg/ml)  
 17 Apo2L (1μg/ml) + Fc (.33μg/ml)  
 18 Apo2L (1μg/ml) + Fc (.11μg/ml)  
 19 Apo2L (1μg/ml) + Fc (.037μg/ml)  
 20 Apo2L (1μg/ml) + Fc (.012μg/ml)  
 21 Apo2L (1μg/ml) + Fc (.004μg/ml)  
 22 Apo2L (1μg/ml) + Fc (.0014μg/ml)  
 23 Apo2L (1μg/ml) + Fc (.0004μg/ml)  
 24 Apo2L (1μg/ml) + medium

## III. TNFR1 IgG (DILUTE 1:10)

- 25 Apo2L (1μg/ml) + Fc (27μg/ml)

- 26 Apo2L (1μg/ml) + Fc (9μg/ml)  
 27 Apo2L (1μg/ml) + Fc (3μg/ml)  
 28 Apo2L (1μg/ml) + Fc (1μg/ml)  
 29 Apo2L (1μg/ml) + Fc (.33μg/ml)  
 30 Apo2L (1μg/ml) + Fc (.11μg/ml)  
 31 Apo2L (1μg/ml) + Fc (.037μg/ml)  
 32 Apo2L (1μg/ml) + Fc (.012μg/ml)  
 33 Apo2L (1μg/ml) + Fc (.004μg/ml)  
 34 Apo2L (1μg/ml) + Fc (.0014μg/ml)  
 35 Apo2L (1μg/ml) + Fc (.0004μg/ml)  
 36 Apo2L (1μg/ml) + medium

5.2 + 10.4 MEDIUM, TAKE OUT 10.4

30.8μl Apo2L  
14μl + 15μl Fc = 1μg/ml

X = 8.1 X 12 = 97.2

9.1 + 18.2 MEDIUM ⇒ TAKE OUT 18.2

ROTATE MULTIPLE TIMES

6.75 + 13.5 ⇒ ADD 13.5

1. 6.9%	13. 9.8%	25. 46.6%
2. 8.1%	14. 8.8%	26. 46.4%
3. 6.7%	15. 9.1%	27. 52.1%
4. 6.7%	16. 10.4%	28. 39.2%
5. 10.2%	17. 20.9%	29. 47.0%
6. 15.6%	18. 24.3%	30. 44.5%
7. 20.4%	19. 41.7%	31. 41.3%
8. 26.3%	20. 46.6%	32. 50.5%
9. 33.8%	21. 39.4%	33. 52.8%
10. 41.0%	22. 49.5%	34. 54.4%
11. 42.8%	23. 49.2%	35. 45.4%
12. 49.0%	24. 54.5%	36. 39.2%

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

NOTEBOOK NO. 26865  
ISSUED TO Scot Masters  
ON [REDACTED] 19 [REDACTED]  
DEPARTMENT Molecular Oncology  
RETURNED 19

—SCIENTIFIC NOTEBOOK CO.—  
2831 LAWRENCE AVE.  
P.O. BOX 238  
STEVENSVILLE, MI 49127  
616-429-8285

ADE- 24  
USSN 10/052,798

From Page No. \_\_\_\_\_

Set up TX of 293S cells. 100mm Dishes.  
Fed cells 8:00am.

Tube A

0.5 ml's 1:10 TE  
50  $\mu$ l 2.5M CaCl<sub>2</sub>  
1  $\mu$ l V RNA  
10  $\mu$ g DNA Below.

Tube B

0.5 ml's 2X HBS

DNA on cells 10:00am  
Fed Fresh media 4:00pm

Plate #

DNAs

1	PRKS	
2	HVEM	
3	TNFR1	
4	Apo-3	
5	PRKS	} 2 $\mu$ g + Ap-1 Luciferase
6	HVEM	
7	TNFR1	
8	APO-3	
9	PRKS	} 2 $\mu$ g + NFkB Luciferase
10	HVEM	
11	TNFR1	
12	Apo-3	
13	PRKS	} Inocyte 2098364 1237537
14	DD 1.1	
15	" 2.1	
16	" 3.1	
17	" 4.1	

For C-Jun (SAPK Assay)

Go To pg # 51

For Luciferase.  
Below.

Gave To Jamie  
For Facs.

# 2.1 kills cells.

Harvest Luciferase cells 5-12 in 1ml  
Cell lysis Buffer. put on rotator 30 min  
& Count on Luciferase Assay.  
To pg # 49

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Recorded by

From Page No. \_\_\_\_\_

Northerns From May 9  
OR New D.D. Receptor.  
2.1

Witnessed &amp; Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

northern  
1hr  
exposure

Kidney  
Liver  
Lung  
Brain

PBLs  
Colon  
sm intesti  
Ovary  
Testis  
prostate  
Thymus  
Spleen

pancreas  
Kidney  
SK MUSC  
Liver  
Lung  
placenta  
Brain  
Heart

dition JRM

From Page No.     

TX OF 293 cells Fed 12:00 noon

Tube #

DNA

1

PrFS

DNA on cells

2

Apo-3-Ig6 #31

# 4:00 pm

3

DD.2-Ig6 #5

4

DD.2-Ig6 #20

5-15

DD.2-Ig6 #6

16-26

DD.2-Ig6 #20

plates 1-4 Labeled with  
2.5 ml/s met-cys. media  
+ 0.3 mic. Pr mix  
S<sup>35</sup> met + cys

8:50 am.

plates 5-26

Fed  
S.F. media

noon.

Harvest sups off plates 1-4

3:00 pm.

3/18/97

Harvest S.F. sup off plates

Hot sups  
pg #66To Page No.     Witnessed & Understood by me,     Date     Invented by     Date     Recorded by

Project No. \_\_\_\_\_  
Book No. 26865

65

**TLE.**

rom Page No.\_\_\_\_

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date \_\_\_\_\_

**Invented by**

Date \_\_\_\_\_

Recorded by \_\_\_\_\_



From daemon [REDACTED]

Date: [REDACTED]

To: sam@gene.COM

Subject: rhserver@shgc: completed two point calculations

messageID: [REDACTED].OAA25204.

reference: dd.2

The following information contains the SHGC framework marker which best links with your marker with a LOD score of 6 or greater. Note that if you want to consider LOD scores lower than 6, you must provide a chromosomal assignment for your marker.

The results of this query are designed to be used in conjunction with the G3 maps found on the SHGC web page. Please visit our RHMmapping section at <http://www-shgc.stanford.edu/rh/frames/engine.html> and query with the name of the linked marker for information about map position and other markers in the area.

For reference, your original email submission is appended to these results.

Disclaimer: Neither the Stanford Human Genome Center nor Stanford University make any claims about veracity or suitability of these results. This information is provided on an as-is basis only.

Two Point Maximum Likelihood Analysis results for submitted marker dd.2.

The raw scoring data was:

```
000000000000000000000000100100000010000011010011000100010000R00000100000000001000:
000
```

The calculation results were:

<u>submitted marker</u>	<u>linked marker</u>	LOD	cR_10000	chrom
-------------------------	----------------------	-----	----------	-------

Reporting best lod  $\geq 6.0$ 

dd.2	D8S481	11.054567	9.90	8
------	--------	-----------	------	---

Original submission:

>From nobody [REDACTED]

```
>Return-Path: <nobody>  
>Received: by shgc.Stanford.EDU (8.7.4/inc-1.0)  
> id OAA25204; [REDACTED]  
>Date: [REDACTED]  
>From: nobody (uid no body)  
>Message-Id: [REDACTED] OAA25204@shgc.Stanford.EDU>  
>To: rhserver@shgc.stanford.edu  
>Subject: submission  
>  
>//  
>exp_text: dd.2  
>//  
>contact_email: sam@gene.com  
>rh_name: dd.2  
>rh_score: 0000000000000000000000000100100000010000011010011000100010000R000001000  
00000001000000  
>//
```

From Page No. \_\_\_\_\_

3/17/97 Set up R.p.s of Sups From  
[REDACTED] pg # 62.200 $\mu$ L SUP  
+ 200 $\mu$ L 2X R.p.s (Pre washed 293  
+ 15 $\mu$ L Pancreatin (Pre inc with cold lysate 30 min)

30 min 4°

wash 2X 1X R.p.s  
wash 1X dH<sub>2</sub>O.Add 15 $\mu$ L 1X Sample Buffer NO DTT.

Boil 5 min.

Load Gel.

/ Dye / Dye / p.r.k.s / App. 3 / DD. 2 / DD. 2 / M / Dye / Dye / Dye /  
IgG IgG IgG  
#6 #20

Stain

De stain

Enhance

dH<sub>2</sub>O

Vac Dry &amp; expose.

Witnessed &amp; Understood by me,

Date

Invented by

Date

Recorded by

Martex

00.2-Ig6  
#20

00.2-Ig6  
#6

Apo-3 Ig6

PKS

3/17/97  
hr.





3/18/97

C.V.  
exposure.

cp317

TITLE \_\_\_\_\_

From Page No. \_\_\_\_\_

3/18/97 Took 1.5 ml's of sup from pg # 62 cold

PKS

DD.2-IgG #6

DD.2-IgG #20

Add 30  $\mu$ L of Protein A Sepharose.

2hr RT on Rotator.

Wash 3x PBS

Split to Two Tubes

Add Sample Buffer to 1 set <sup>NO DTT</sup>  
" " " + DTT to 1 set.

Box 1 S'

Load Gel.

NO DTT				+ DTT			
empty	PKS	DD.2 IgG #6	DD.2 IgG #20	empty	PKS	DD.2 IgG #6	DD.2 IgG #20
							M
							empty

Transfer to nitrocellulose &

put in ~~power~~ 5% M. TK + PBST O.IX.  
to Block.

3/19/97 Add Abx Ho-IgG HRP (1:1000) 2hrs

Wash 3x PBST 5min

Develop by chemiluminescence

To Page No. 70

Witnessed & Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

From Page No. 3/18/96sent sups to Assay services for hu PC elisa

## ASSAY

To sign up for assays call x2632  
Drop-off samples in room 5295

ASSAY

TEST PROCEDURE

SAMPLES SUBMITTED BY <u>Scot Marsters</u>	EXTENSION <u>1849</u>	MAILSTOP # <u>410</u>	COST CENTER <u>424</u>	PROJECT CODE <u>1110</u>	PRODUCT ID <u>DD2 IgG</u>	# SAMPLES <u>15 x 2 = 30</u>
DATE SUBMITTED <u>3/18/97</u>	DATE TO BE ASSAYED <u>3/19/97</u>	<input type="checkbox"/> WILL PICK UP <input type="checkbox"/> MAIL RESULTS <input type="checkbox"/> FAX RESULTS (# <u>544</u> ) <input type="checkbox"/> CALL ME		STORAGE TEMP. (PRE-ASSAY) <input type="checkbox"/> AMBIENT <input type="checkbox"/> -10°C OR BELOW <input type="checkbox"/> 2° - 8°C <input type="checkbox"/> OTHER		
<input type="checkbox"/> GMP <input type="checkbox"/> GLP Study No. <u>        </u> <input type="checkbox"/> N/A	SAMPLE MATRIX <u>PLS</u>	ANTICOAGULANT *If used <u>        </u>	<input type="checkbox"/> RADIOACTIVE <input type="checkbox"/> TOXIC HAZARD <input checked="" type="checkbox"/> BIOHAZARD (NON-HUMAN/NON-PRIMATE) Specify type and amount: <u>        </u>	SAMPLE OF HUMAN/PRIMATE ORIGIN? <input type="checkbox"/> NO <input checked="" type="checkbox"/> YES If yes, identify source: <u>293 cells</u> (specify tissue, cell line, blood, etc.)		
SAVE SAMPLES? <input type="checkbox"/> YES: Storage Temp. <u>        </u> <input checked="" type="checkbox"/> NO: Samples not picked up within 2 weeks will be discarded.		NOTES				

## NOTES

Please Run samples in Duplicate400ul/tube

## TIME OF REPORT

DATE

TIME

INITIALS

PLEASE CALL THE LAB WITH ANY QUESTIONS OR PROBLEMS REGARDING ASSAY RESULTS

ASSAY RESULTS ARE NOT CORRECTED FOR DILUTION

NO	IDENTIFY EACH SAMPLE ORIGIN AND/OR UNIQUE COMPONENTS	DILUTION			NO	IDENTIFY EACH SAMPLE ORIGIN AND/OR UNIQUE COMPONENTS	DILUTION		
1	Int Trans	neat			21				
2	"	1:3.3			22				
3	"	1:10			23				
4	"	1:33	1	-0.002	LTS	-0.005	Conc	%CV (Conc)	Mean Conc
5	"	1:100	2	-0.005	LTS	-0.004	LTS	n/a	n/a
6	DD 2 IgG #6	neat	3	-0.004	LTS	-0.005	LTS	n/a	n/a
7	"	1:3.3	4	-0.005	LTS	-0.002	LTS	n/a	n/a
8	"	1:10	5	0.000	LTS	-0.005	LTS	n/a	n/a
9	"	1:33	6	-0.003	LTS	-0.006	LTS	n/a	n/a
10	"	1:100	7	-0.005	LTS	-0.007	n/a	n/a	n/a
11	DD 2 IgG #20	neat	8	-0.006	LTS	-0.005	LTS	n/a	n/a
12	"	1:3.3	9	-0.005	LTS	-0.005	LTS	n/a	n/a
13	"	1:10	10	-0.005	LTS	-0.006	LTS	n/a	n/a
14	"	1:33	11	-0.007	n/a	-0.006	LTS	n/a	n/a
15	"	1:100	12	-0.006	LTS	-0.002	LTS	n/a	n/a
			13	-0.002	LTS	-0.003	LTS	n/a	n/a
			14	-0.002	LTS	-0.004	LTS	n/a	n/a
					33	-0.004	LTS	n/a	
					34				
					35				

To Page No.         

Witnessed &amp; Understood by me,

Date

Invented by

Date

Recorded by

TITLE \_\_\_\_\_

From Page No. \_\_\_\_\_

3/18/97 make W F B Probe

5mL 2505  
3mL 10XLB  
3mL PNC  
9mL dH<sub>2</sub>O  
10mL 8P 32 ATP

32° 45 min

purify on Quickspin

Count 1  $\mu$ L.

USER: 9 ID:32P PRESET TIME: 1.00  
SAMPLE REPEAT: 1 CYCLE REPEAT: 1 SCR:N RS232:N  
H#: 0 AQC:N QCF:N RCM:N  
CHANNEL 1-LL: 0 UL:1000 2SIGMA: 2.00 BKG SUB: 0.00 BKG 2SIG: 0.00 LSR:  
DATA CALC: CPM, UNKNOWN REPLICATES: 1 NORM FACTOR: 0 1.00000  
HALF LIFE(DAYS):N

SAM	PDS	CH	CPM	2SIG%	TIME	EL TIME
1	**	1	1395080.00	0.76	0.05	0.24

$\approx 1.4 \times 10^6$  cpm/ $\mu$ L

To Page No. \_\_\_\_\_

Witnessed & Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_



NFKB

Dose response +

Project No. \_\_\_\_\_

TITLE \_\_\_\_\_

Time Course  
of TNF + Apo2L

Book No. 26865

71

From Page No. \_\_\_\_\_

3/19/97 Used Hela cells set up on 3/18/96

well #	Ligand + Conc.	Time
1	Apo2L 1ng/ml	0
2	"	10 min
3	"	20 "
4	"	30 min
5	"	1 hr
6	"	2 hr
7	TNF 1ng/ml	0
8	"	10 min
9	"	20 min
10	"	30 min
11	"	1 hr
12	"	2 hr
13	Apo2L 0	30 min
14	" 0.1 ng/ml	"
15	" 1 "	"
16	" 10 "	"
17	" 100 "	"
18	" 1000 "	"
19	TNF 0 "	"
20	" 0.1 "	"
21	" 1 "	"
22	" 10 "	"
23	" 100 "	"
24	" 1000 "	"

Set up NFKB assay as usual.  
Gel loading

1	1	2	3	4	5	6	7	8	9	10
2	11	12	13	14	15	16	17	18		
3	19	20	21	22	23	24				

To Page No. 73

Witnessed &amp; Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

From Page No. \_\_\_\_\_

3/19/97 set up Electroporations + HeLa plates  
pK ↓ CRMA ↓ Traf2 O/n PBS ↓ DEVD ↓ ZVAD

Set everything up in Duplicate

well #

3/20 Ligand

Time  
for NFKBTime For  
SAPK

SET UP	1	PBS	PBS	1hr	2hr
	2	"	AP02L 1ug/ml	1hr	2hr
1 X 10 <sup>6</sup> cells per well	3	"	TNF 1ug/ml	20min	15min
then add	4	DEVD 200uM	PBS	1hr	2hr
	5	"	AP02L 1ug/ml	1hr	2hr
	6	"	TNF 1ug/ml	20min	15min
PBS	7	Z VAD	PBS	1hr	2hr
DEVD	8	"	AP02L 1ug/ml	1hr	2hr
Z VAD	9	"	TNF 1ug/ml	20min	15min
STOCK 50mM.					
Electroporation	10	PrK5	PBS	1hr	2hr
1 X 10 <sup>6</sup> cells per well	11	"	AP02L 1ug/ml	1hr	2hr
	12	"	TNF 1ug/ml	20min	15min
with 10ug plasmid	13	CRMA	PBS	1hr	2hr
	14	"	AP02L 1ug/ml	1hr	2hr
Fed 4hrs	15	"	TNF 1ug/ml	20min	15min
later.	16	TraF2 O/N	PBS	1hr	2hr
	17	"	AP02L 1ug/ml	1hr	2hr
	18	"	TNF 1ug/ml	20min	15min

3/20/97 set up 1 set For NFKB  
Go to pg # 743/20/97 set up 1 set For SAPK Assay  
pg # 75

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Recorded by

Book No. ~~26865~~

# TITLE

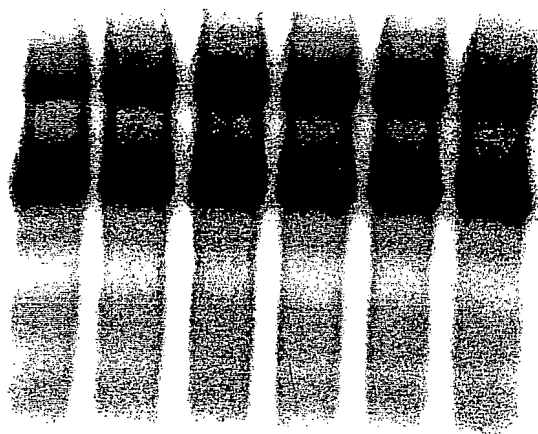
From Page No. \_\_\_\_\_

**Recorded by**

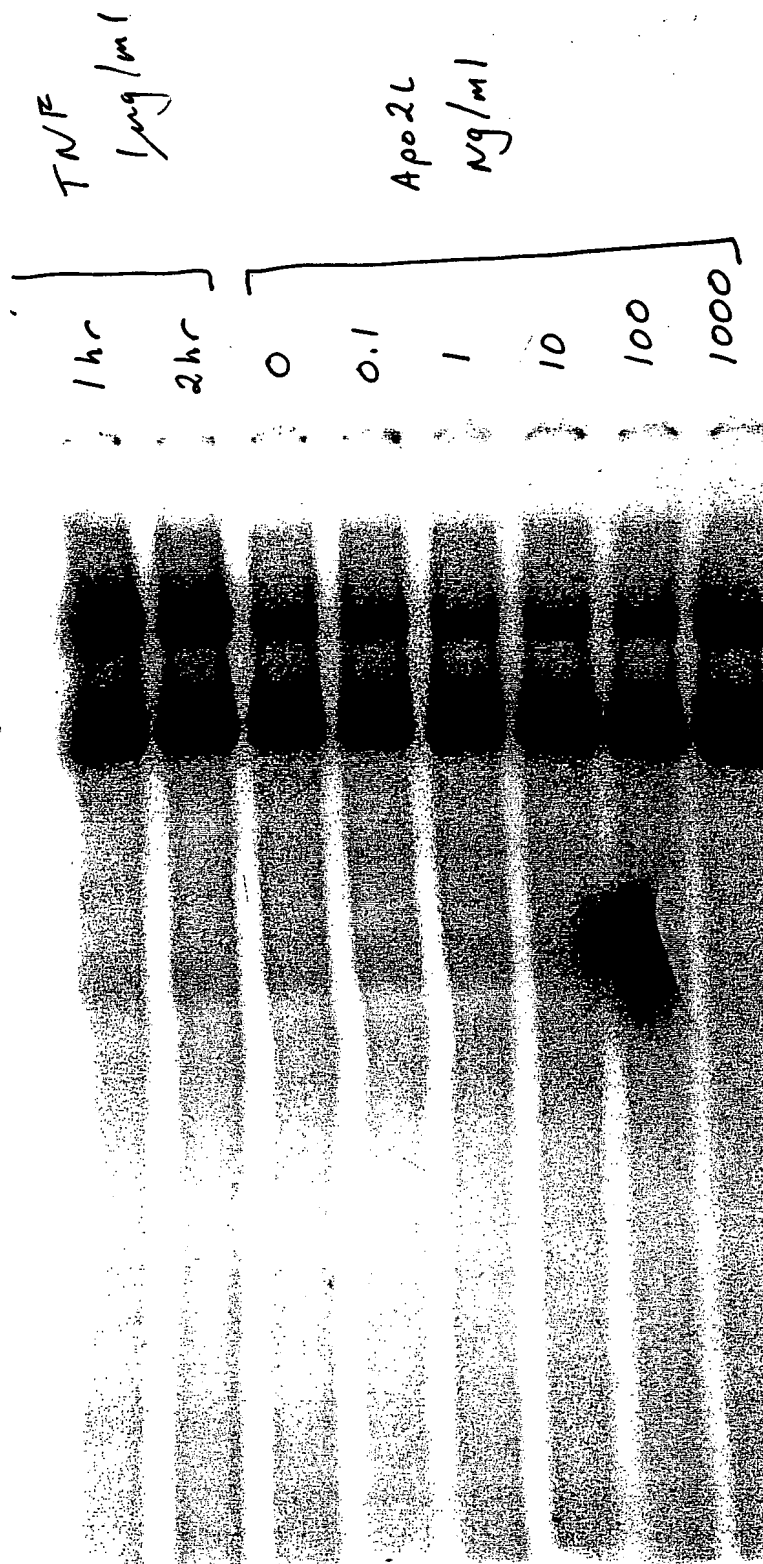
3/20/97

TNF  
ng/ml.

0 0.1 1 10 100 1000



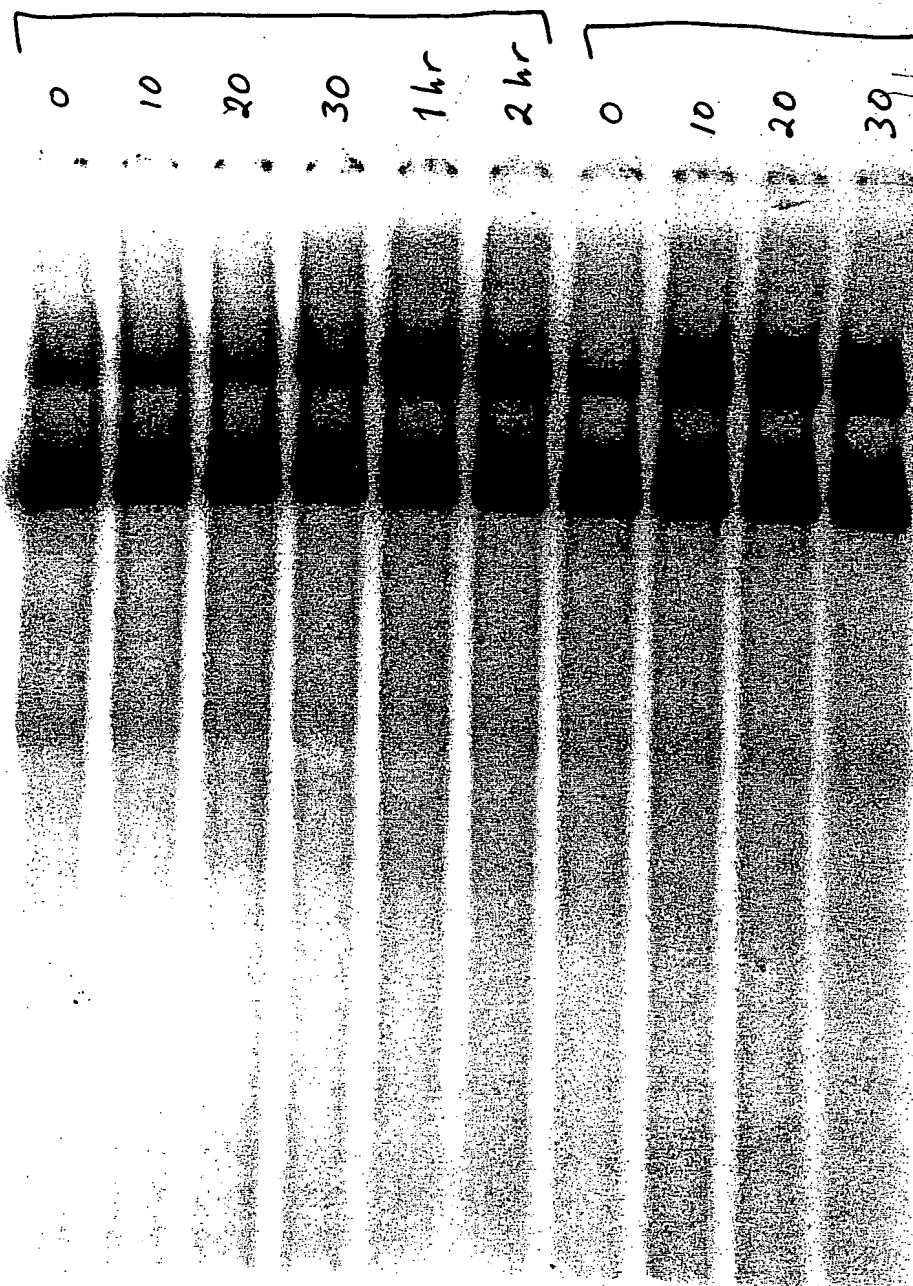
3/20/97

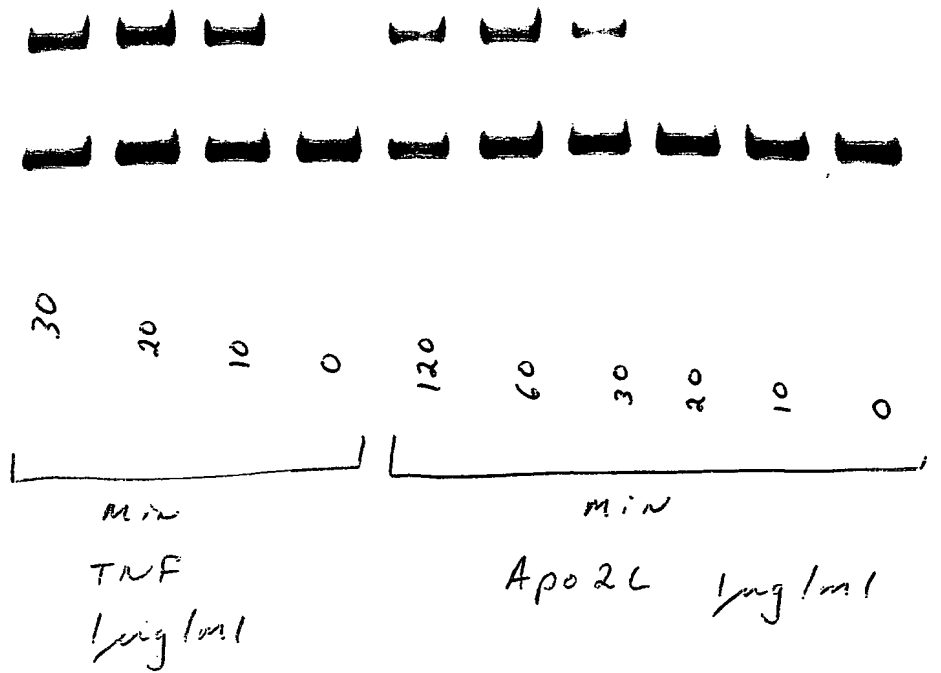
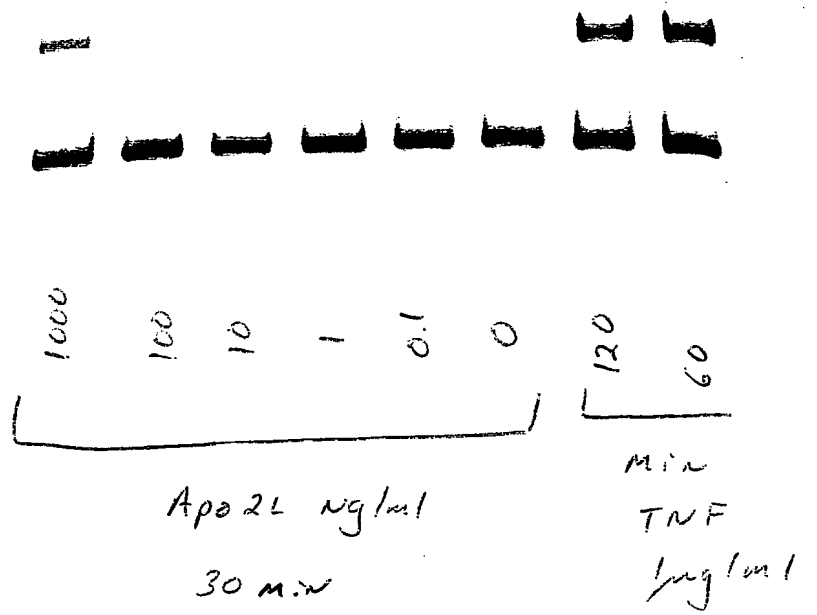


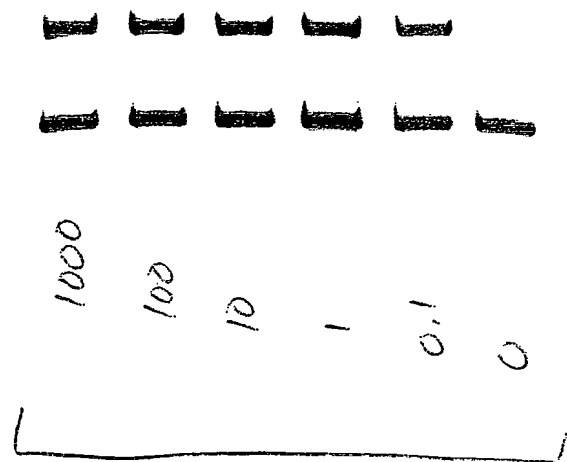
3/20/97

Apo2C  
1  $\mu$ g/ml

TNF  
1  $\mu$ g/ml







TNF ng/ml

30 min



From Page No. \_\_\_\_\_

3/21/97 Used HeLa cells set up 3/20/97 6 well  
Dishes.

	PreTreat	Ligand	conc	Time
1	PBS	PBS		
2	"	Apo2L	1 ng/ml	30 min
3	"	TNF	1 ng/ml	"
4	ALLN (100 ng/ml) 2 hr	PBS		
5	"	Apo2L	1 ng/ml	30 min
6	"	TNF	1 ng/ml	"
7	Cyclohexanide (500 ng/ml) 2 hr	PBS		
8	"	Apo2L	1 ng/ml	30 min
9	"	TNF	1 ng/ml	"
10	none	TNF	1 ng/ml	0
11		"		10 min
12		"		20 min
13		"		30 min
14		"		1 hr
15		"		2 hr
16		LT	20 ng/ml	0
17		"		10 min
18		"		20 min
19		"		30 min
20		"		1 hr
21		"		2 hr
22		LT	0	30 min
23		"	0.1 ng/ml	"
24		"	1 ng/ml	"
25		"	10 ng/ml	"
26		"	100 ng/ml	"
27		"	1000 ng/ml	"

IN  
DuplicateFor NF $\kappa$ B assay OF 1-27 go to pg # 77  
For SAPK assay OF 10-27

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Recorded by

Project No. \_\_\_\_\_  
Book No. 26865

77

TITLE \_\_\_\_\_

From Page No. \_\_\_\_\_

3/2/97

NFKB Assay

Set up as usual

To Page No. \_\_\_\_\_

Witnessed & Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

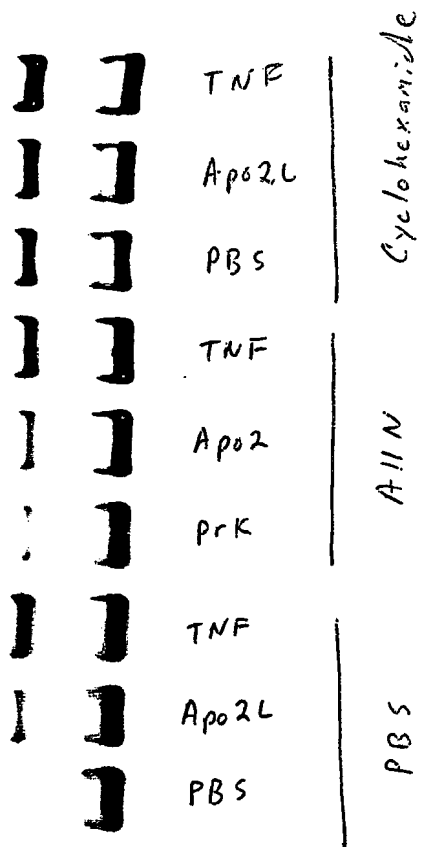
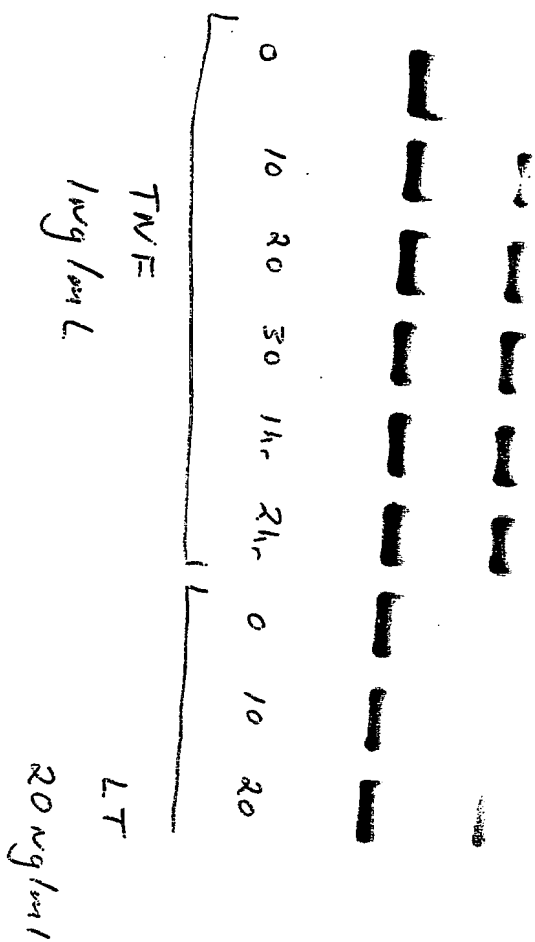
Recorded by \_\_\_\_\_

一、  
 二、  
 三、  
 四、  
 五、  
 六、  
 七、  
 八、  
 九、  
 十、

LT  
kg/m<sup>3</sup>

3/22/97

3/22/97



3/22/97

TX OF  
DD.2-Ig6.

Project No. 26865  
Book No. 26865

87

TLE

From Page No.

3/28/97

used 35 100MM Dishes of 293S  
cells Fed cells 11:00 a.m.

Set up 10 plate each of + 5 plates of  
DD.2 Ig6 #10 Mock TX  
" #31  
" #41

Tube A  
5mls 1:10 TE  
0.5mls 2.5M CaCl<sub>2</sub>  
10µL VARNA  
100µg DNA above.

Tube B  
5mls 2X HBS

DNA on cells 4:00 p.m.

3/29/97

Fed cells Serum Free media

3/31/97 signed up for FC ELISA Assay on  
pg # 90.

To Page No. 90

Witnessed & Understood by me,

Date

Invented by

Date

Recorded by

From Page No. \_\_\_\_\_

**ASSAY REQUEST/REPORT FORM**  
**Genentech, Inc.**ASSAY: Fe Tit

To sign up for assays call x2632.

Drop off samples in room 5295.

**GREY TO BE COMPLETED BY ASSAY SERVICES**

A.S. EXPT NO.		DATE	
A.S. TECH		TEST PROCEDURE	
SAMPLES SUBMITTED BY		# SAMPLES	
EXTENSION		PRODUCT I.D.	
MAILSTOP #	COST CENTER	PROJECT CODE	
40	424	1110	DD.2-Tg6
DATE SUBMITTED	DATE TO BE ASSAYED	STORAGE TEMP. (PRE-ASSAY)	
3/31/97	3/31/97	<input type="checkbox"/> AMBIENT <input type="checkbox"/> -10°C OR BELOW <input checked="" type="checkbox"/> 2° - 8°C <input type="checkbox"/> OTHER	
<input type="checkbox"/> GMP <input type="checkbox"/> GLP Study No. _____ <input type="checkbox"/> N/A		<input type="checkbox"/> WILL PICK UP* <input type="checkbox"/> E-MAIL RESULTS (Log In: _____) <input type="checkbox"/> MAIL RESULTS <input type="checkbox"/> FAX RESULTS (# _____) *Results not picked up by end of week are mailed.	
SAMPLE MATRIX		<input type="checkbox"/> RADIOACTIVE <input type="checkbox"/> TOXIC HAZARD <input checked="" type="checkbox"/> BIOHAZARD (NON-HUMAN/NON-PRIMATE) Specify type and amount: <u>293 cells</u>	
ANTICOAGULANT		SAMPLE OF HUMAN/PRIMATE ORIGIN? <input type="checkbox"/> NO <input checked="" type="checkbox"/> YES	
*if used.		If yes, identify source: <u>293 cells</u> (specify tissue, cell line, blood, etc.)	
SAVE SAMPLES?		NOTES:	
<input type="checkbox"/> YES: Storage temp. _____ <input type="checkbox"/> NO: Samples not picked up within 2 weeks will be discarded.			

**ASSAY RESULTS ARE NOT CORRECTED FOR DILUTION**

NO.	IDENTIFY EACH SAMPLE ORIGIN AND/OR UNIQUE COMPONENTS	DILUTION	NO.	IDENTIFY EACH SAMPLE ORIGIN AND/OR UNIQUE COMPONENTS	DILUTION
1	# 10	Neat	21	# 411	Neat
2	"	1:10	22	"	1:10
3	"	1:100	23	"	1:100
4	"	1:1000	24	"	1:1000
5	# 10	Neat	25	untreated	Neat
6	"	1:10	26	"	1:10
7	"	1:100	27	"	1:100
8	"	1:1000	28	"	1:1000
9	# 31	Neat	29		
10	"	1:10			
11	"	1:100			
12	"	1:1000			
13	# 31	Neat			
14	"	1:10			
15	"	1:100			
16	"	1:1000			
17	# 411	Neat			
18	"	1:10			
19	"	1:100			
20	"	1:1000			

#	O.D.	Conc	#	O.D.	Conc
1	-0.008	LTS	15	-0.005	LTS
2	-0.008	n/a	16	-0.006	L
3	-0.008	n/a	17	0.066	1.
4	-0.007	LTS	18	-0.009	n/a
5	-0.009	n/a	19	-0.009	n/a
6	-0.006	LTS	20	0.109	1.6
7	-0.004	LTS	21	-0.009	n/a
8	-0.004	LTS	22	-0.006	LTS
9	0.002	LTS	23	-0.006	LTS
10	-0.007	LTS	24	-0.005	LTS
11	-0.007	LTS	25	-0.007	LTS
12	-0.006	LTS	26	-0.009	n/a
13	-0.007	LTS	27	-0.007	LTS
			28	-	

Witnessed &amp; Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

TITLE \_\_\_\_\_

From Page No. \_\_\_\_\_

3/31/97 Set up Tx of 2935 cells.  
60mm Dishes. Fed cells 11:00am.

plate #

1	PrKS	
2	DD.2	
3	TNPR1	
4	PrKS	+ DVED 200 $\mu$ M
5	DD.2	
6	TNPR1	
7	PrKS	+ ZVAD 200 $\mu$ M
8	DD.2	
9	TNPR1	
10	PrKS	+ CRMA <del>WAVES</del>
11	DD.2	
12	TNPR1	
13	PrKS	+ h FADD D/N
14	DD.2	
15	TNPR1	

Tube A

0.5 mL 1:10 T.E.  
50  $\mu$ L 2.5 M  $CaCl_2$   
1  $\mu$ L VARN  
10  $\mu$ g DNA above.  
10  $\mu$ g of CRMA or h Fadd D/N if necessary.

Tube B

0.5 mL 2 X HBS

Add 300  $\mu$ L / plate

DNA on cells 4:00 pm.

To Page No. 92

Witnessed & Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

From Page No. 91

4/1/97 worked up samples for NFkB Assay As usual.

## Samples

			NFkB Probe	
1	PKS			
2	DD.2		"	
3	TNFR1		"	
#1B	PKS	From #1	"	+ 2ul Cold
#2B	DD.2	From #2	"	"
3B	TNFR1	From #3	"	"
1C	PKS	From #1	"	+ 2ul $\alpha$ NFkB AB
2C	DD.2	From #2	"	"
3C	TNFR1	From #3	"	"
4	PKS	} + DVED	"	
5	DD.2		"	
6	TNFR1		"	
7	PKS	} + ZVAD	"	
8	DD.2		"	
9	TNFR1		"	
10	PKS	} + CRMA	"	
11	DD.2		"	
12	TNFR1		"	
13	PKS	} + h Padd	"	
14	DD.2		"	
15	TNFR1		"	D/N

Gel 1

1/2/3/1B/2B/3B/1C/2C/3C

Gel 2

4/7/5/6/8/9/10/11/12

Gel 3

13/14/15

To Page No. 92

Witnessed &amp; Understood by me,

Date

Invented by

Date

Recorded by



Project No. \_\_\_\_\_  
Book No. 26865

93

TITLE \_\_\_\_\_

From Page 93

From Page 93

Witnessed & Understood by me,

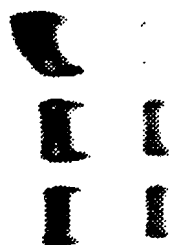
Date \_\_\_\_\_

Invented by

Date \_\_\_\_\_

Recorded by

13 14 15



040297.b.1

From Page No. \_\_\_\_\_

4/14/97 Label probe For NF $\kappa$ B expt.5  $\mu$ L 01.90 25053  $\mu$ L 10x CB3  $\mu$ L Pnk9  $\mu$ L dH<sub>2</sub>O10  $\mu$ L P32 ATP.

45 min 37°

purify on Quick spin  
columnCount 1  $\mu$ L.

USER: 9 ID:32P

PRESET TIME: 1.00

SAMPLE REPEAT: 1 CYCLE REPEAT: 1 SCR:N RS232:N

H#: 0 ADC:N QCF:N RCM:N

CHANNEL 1-LL: 0 UL:1000 2SIGMA: 2.00 BKG SUB: 0.00 BKG 2SIG: 0.00 LSR: 0

DATA CALC: CPM, UNKNOWN REPLICATES: 1 NORM FACTOR: 0 1.00000

HALF LIFE(DAYS):N

SAM	POS	CH	CPM	2SIG%	TIME	EL TIME	ERR
1	**	1	1700940.00	0.69	0.05	0.24	

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

2935 TX

Project No. \_\_\_\_\_  
Book No. 26865

95

TITLE \_\_\_\_\_

From Page No. \_\_\_\_\_

4/14/97 Set up TX 100mm Dishes.  
Fed cells 9:00 am.

Plate #

1	PrK 5
2	DD.2
3	TNFR1
4	PrK 5
5	DD.2
6	TNFR1
7	PrK 5
8	DD.2
9	TNFR1

+ Z VAD

+ PrK  
CRMA.

Tube A

0.5 ml/s 1:10 TE  
50  $\mu$ l 2.5 M CaCl<sub>2</sub>  
10  $\mu$ g DNA  
1  $\mu$ g VARNA.

Tube B

0.5 ml/s 2x HBs.

DNA on cells 12:00 noon.

Jamie Fed cells Fresh med. g  
at 9:00 pm. + put ZVAD on  
plates 4-6.

4/15/96.

Set up NFkB Assay as usual.

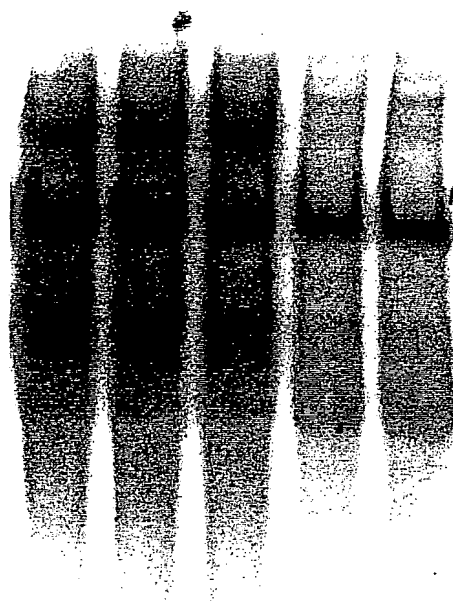
Sample 1-3 also set up tube's labeled

A 60T 3  $\mu$ l Cold Oligo

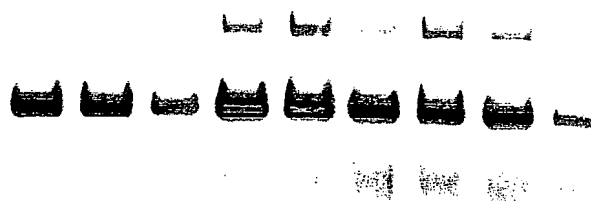
B 60T 3  $\mu$ l  $\alpha$  NFkB AB  
15 min after START OF  
incubation.

No. \_\_\_\_\_

Recorded by \_\_\_\_\_



1 2 3 1A 2A



3A 1B 2B 3B 4 5 6 7 8 9

From Page No. \_\_\_\_\_

4/14/97 Set up Electroporations in Hela cells  
 $1 \times 10^6$  cells/tube.

Plate #

1 PRKS

2 DD.2

3 TNFR1

4 PRKS

5 DD.2

6 TNFR1

7 PRKS

8 DD.2

9 TNFR1

+ ZVAD

+ PRK  
CRMA

DNA on cells

1:00 pm

reFed cells

3:00 pm +

Add ZVAD to  
Tubes 4-6

4/15/97 Set up NFKB as usual.

samples 1-3 also set up as

A Got 3ul cold oligo

+

B Got 3ul  $\alpha$  NFKB AB

15 min after start of

Incubation.

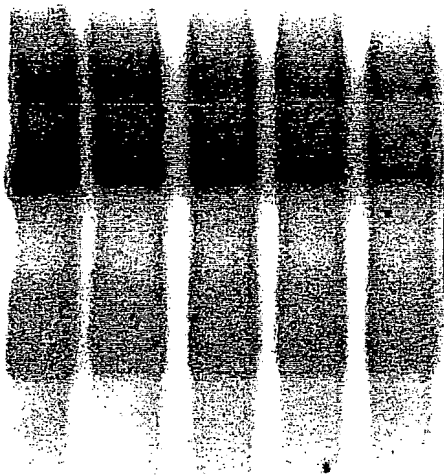
Witnessed &amp; Understood by me,

Date

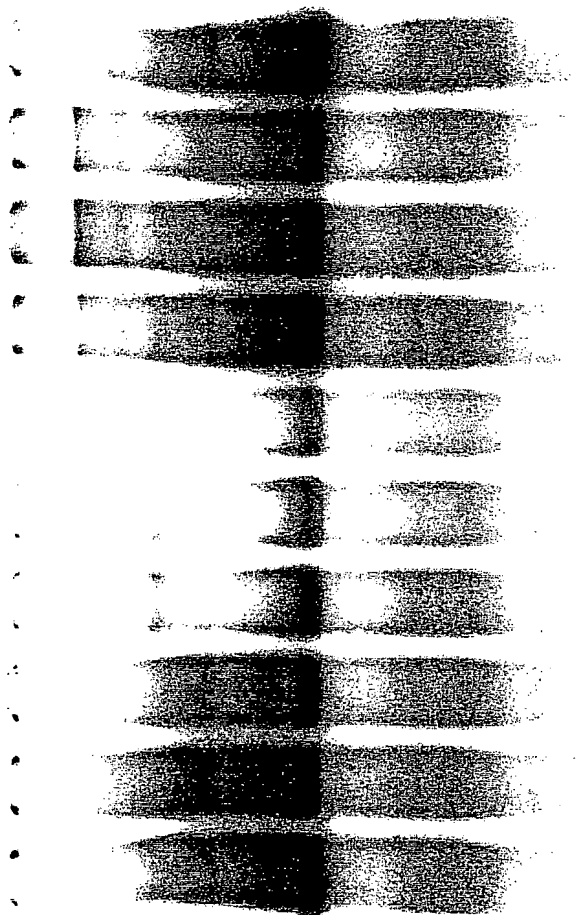
Invented by

Date

Recorded by



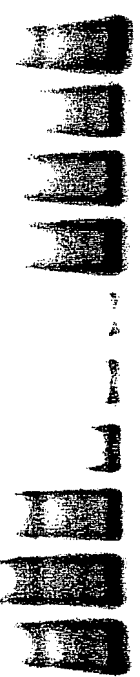
H	H	H	H	H
5	6	7	8	9



H	4
H	3C
H	2B
H	1B
H	3A
H	2A
H	1A
H	3
H	2
H	1



TNFR1	+ CRMA
DD.2	
PrK	
TNFR1	+ Z.VAD.
DD.2	



PrK	+ 2
TNFR1	
DD.2	
PrK	+ 2
TNFR1	
DD.2	+ Cold
PrK	
TNFR1	
DD.2	
PrK	

He 19



NOTEBOOK NO. 26119  
ISSUED TO Scot Masters  
ON [REDACTED] 19 [REDACTED]  
DEPARTMENT Mol. Onc.  
RETURNED 19

—SCIENTIFIC NOTEBOOK CO.—  
2831 LAWRENCE AVE.  
P.O. BOX 238  
STEVENSVILLE, MI 49127  
616-429-8285

ADE- 25  
USSN 10/052,798

From Page No. \_\_\_\_\_

set up PCRs.

0.5  $\mu$ L DNA  
10  $\mu$ L 10x PCR Buffer  
24  $\mu$ L 1.25 mM dNTPs  
1  $\mu$ L 5' primer  
1  $\mu$ L 3' primer  
63  $\mu$ L dH<sub>2</sub>O  
1  $\mu$ L Taq

+ 0.1

3' 94°  
1 cycle

1' 94°  
1' 50°  
3' 72°  
16 cycles

run 0.1V.

DNA

- 1) DD 2.1
- 2) "
- 3) "
- 4) DDK 4.1
- 5) "
- 6) "

primers

5'	3'
prK rev	SM.3
SM.1	"
SM.2	"
prK rev	"
SM.1	"
SM.2	"

set up Digest

Add 10  $\mu$ L ~~100 mM~~ 10x RB

+ 2  $\mu$ L BSTE II

55° 2 hr

Then Add 2  $\mu$ L RI

37° 0.1V.

Digest Vector.

83  $\mu$ L water  
3  $\mu$ L DNA  
10  $\mu$ L 10x RB 100 mM  
+ 2  $\mu$ L BSTE II  
+ 2  $\mu$ L RI

37° 0.1V.

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

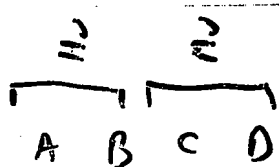
Invented by

Date

Recorded by

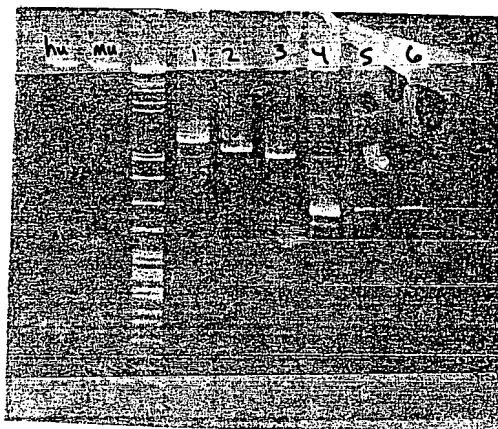
From Page No. \_\_\_\_\_

ran Agarose gel on vectors.



purified vectors with  
Qia quick  
+  
purified PCRs with  
promega kit.

Then Ran Aliquots on  
Gel.



Setup ligations of  
(A+B) (C+D)  
hu + mu & also  
previous cut vector. (E)

with Fragments 1, 2 & 3  
use rapid ligation K.T.

1μL vector  
2μL insert  
2μL vial 2  
10μL vial 1  
mix  
~~5min RT~~  
1μL vial 3  
5min RT

Transformed into JM109's

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

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From Page No. \_\_\_\_\_

Back From Asilomar.

4/10/97

Before we left Maya set up PCRs of  
DD.2 ECD with SM.1 or SM.2 + SM.4

1	SM.1	SM.4	DD.2	Diluted 1:10
2	" .2	SM.4	"	"
3	" .1	SM.4	"	1:100
4	" .2	SM.4	"	"

I took PCRs above + Ran a Gel



I digested  
#3 + #4 with.

90µL PCR

10µL 10X RB 100mM

2µL BSTE II

~~2µL~~

55° 3hr.

+ 2µL RI

+ 2µL PVU I

37° O.N.

Jamie put  
in  
Freezer.

Also I ordered primers to make DD.2-ECD-Flag Tag  
will be ready tomorrow afternoon.

4/11/97 Jamie digested vectors for me.

NB # 27250 pg #27

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

Recorded by

From Page No. \_\_\_\_\_

4/13/97 PCR's. OF DD.2 ECD. Flag.

1  $\mu$ L DNA  
 10  $\mu$ L 10X PCR Buffer  
 24  $\mu$ L 1.25 mM dNTP's  
 1  $\mu$ L 5' primer SM.1 or SM.2  
 1  $\mu$ L 3' primer DD.2 Flag ECD  
 63  $\mu$ L dH<sub>2</sub>O.  
 1  $\mu$ L Tag.

Tube #

Tube #	SM.1	DD.2 Flag ECD
1	SM.1	DD.2 Flag ECD
2	SM.2	"
3	SM.1	"
4	SM.2	"

DNA

DD.2 neat
" "
" 1:10
" "

16 cycles

1' 94°  
 1' 60°  
 2' 72°

That afternoon Tamie Added

10  $\mu$ L 10X RB 100 mM  
 + 2  $\mu$ L R1  
 + 2  $\mu$ L H3

37° O/N,

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

TITLE \_\_\_\_\_

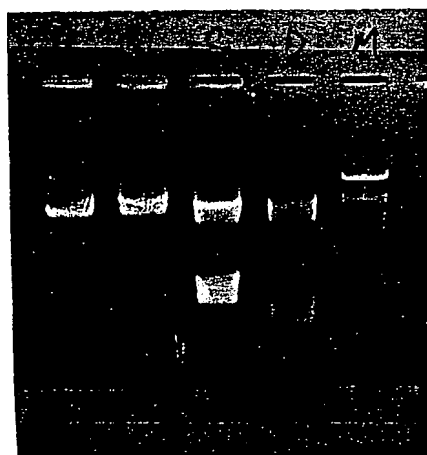
From Page No. \_\_\_\_\_

Agarose.

4/14/97 Ran Gel on vectors.

A <sup>PKS</sup> Hu IFN $\gamma$ R-IgG  
B " Mu " "  
C " Hu " "  
D " Mu " "

Digested w. Th.  
R1-BSTEII  
" "  
R1-H3  
" "



Cleaned up Gel Fragments  
with Qiaquick  
Gel extraction kit.

Cleaned up PCRs From  
4/10 + 4/13 with.

Qiaquick PCR Purification

Set up ligations. Quick

PKS Hu IFN $\gamma$ R-IgG R1-H3  
" Mu " "

+ 1-4 From 4/13  
1-4 " "

2  $\mu$ L Tube 2  
1  $\mu$ L vector  
2  $\mu$ L Frag  
Mix  
+ 10  $\mu$ L Tube 1  
Mix  
1  $\mu$ L Tube 3  
5  $\mu$ L RT  
+ Transform

PKS Hu IFN $\gamma$ R-IgG R1-BSTEII + 3+4 From 4/10

Transformed into JM109s

4/15/97 picked 1-10 colonies DD.2-IgG.

11-82 " DD.2-Flag-ECD

To Page No. 46.

Witnessed & Understood by me,

Date

Invented by

Date

Recorded by

From Page No. \_\_\_\_\_

4/15/97 Setup PCRs of DD.2 IgG or FLAG-ECD

①

x12

20 $\mu$ L dH <sub>2</sub> O + Colony	—
5 $\mu$ L PCR Buffer	60
12 $\mu$ L 1.25 mM dNTPs	144
0.5 $\mu$ L U IgG P4	6
0.5 $\mu$ L PTK R	6
12 $\mu$ L dH <sub>2</sub> O	144
0.5 $\mu$ L Taq	6

②

x80

20 $\mu$ L dH <sub>2</sub> O + Colony	—
5 $\mu$ L PCR Buffer	400
12 $\mu$ L 1.25 mM dNTPs	960
0.5 $\mu$ L PIKF	40
0.5 $\mu$ L PIKR	40
12 $\mu$ L dH <sub>2</sub> O	960
0.5 $\mu$ L Taq	40

1 cycle

940 3'

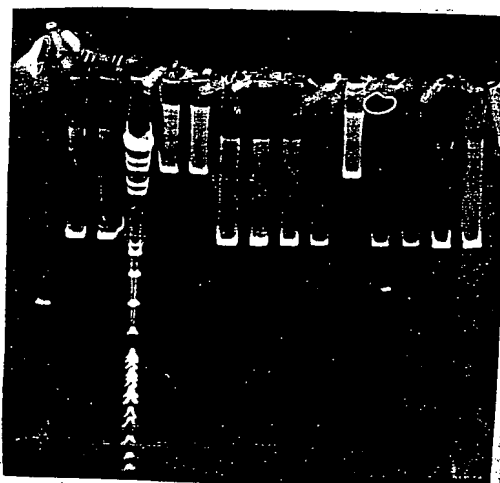
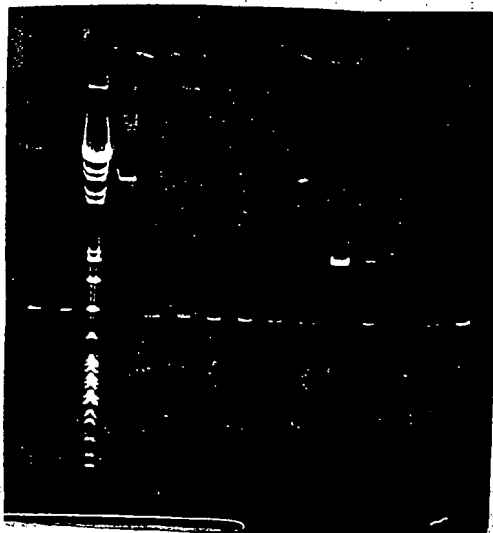
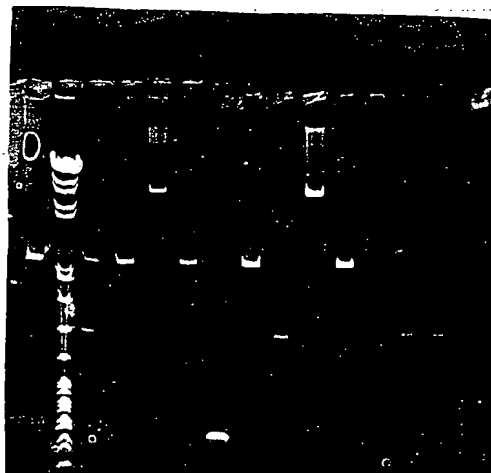
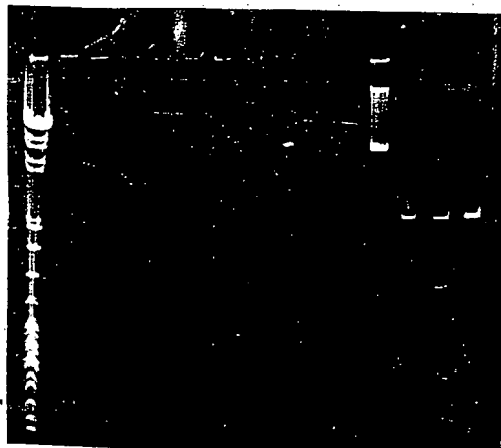
ran out on Gels

30 cycles

1' 940

1' 500

2' 720



Witnessed &amp; Un

Invented by

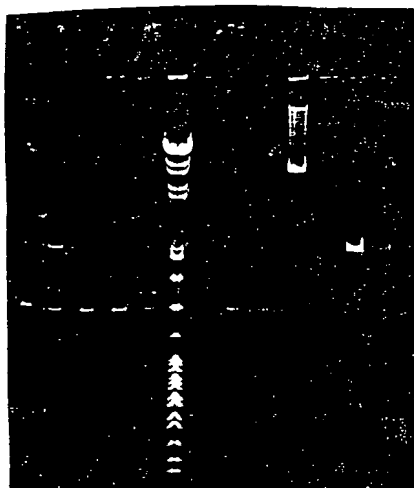
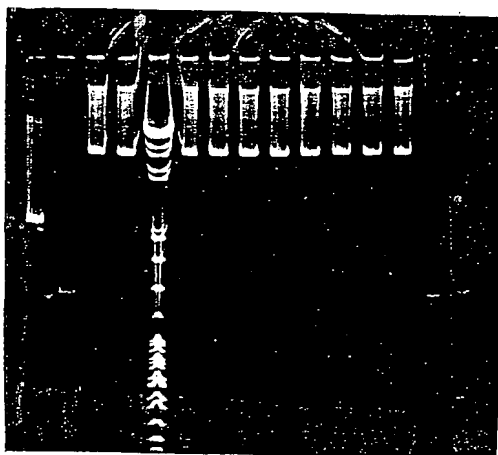
Date

Recorded by

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Ig6's Didn't work

Flag ECD DD.2 worked picked # 21, 38, 56  
and 81 as plasmid preps.

4/16/97 worked up Plasmid preps.

Set up Digests of Plasmids to make Ig6 - DD2 intermediate.  
Then Do mutagenesis.

(1330 Bp)

(1320 Bp)

(1050 Bp)

(800 Bp)

3  $\mu$ L DD.2  
10  $\mu$ L 10x RB 75mM  
2  $\mu$ L RI  
2  $\mu$ L XBA  
83  $\mu$ L dH<sub>2</sub>O

3  $\mu$ L IL-1A Ig6  
10  $\mu$ L 10x RB 75mM  
2  $\mu$ L XBA  
2  $\mu$ L H3  
83  $\mu$ L dH<sub>2</sub>O

3  $\mu$ L DD.2  
10  $\mu$ L 10x RB 75mM  
2  $\mu$ L RI  
2  $\mu$ L SacI  
83  $\mu$ L dH<sub>2</sub>O

3  $\mu$ L 2FC1  
10  $\mu$ L 10x RB 75mM  
2  $\mu$ L SacI  
2  $\mu$ L HindIII  
83  $\mu$ L dH<sub>2</sub>O

37° 2hrs

37° 2hrs

37° 2hrs

37° 2hrs.

run 1% Agarose Gel

To Page No. \_\_\_\_\_

Witnessed & Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by \_\_\_\_\_

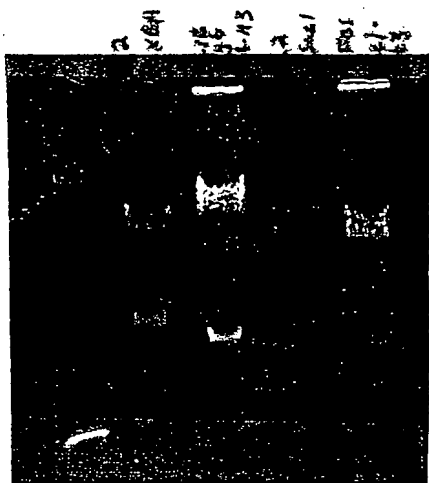
Date \_\_\_\_\_

Recorded by \_\_\_\_\_



From Page No. \_\_\_\_\_

4/16/97



Cut out Bands marked  
+ purified with

Qiagen Qiaquick

Gel  
Extraction  
K.T

Set up Ligations.

(Rapid Ligation K.T)

(C) PrK <sup>HuIPN8RIg6</sup> Δ(RI-H3)  
or

+ either

DD.2 RI-XBA and ILIRIg6 XBA-H3

(D) PrK MuIPN8RIg6  
Δ(RI-H3)

DD.2 RI-SacI and 2PCI SacI-H3

Transformed into JM109S

4/16/97 Digest Plasmid Preps of DD.2 Eco Flag.

1μL DNA  
2μL 10X RB 25mM  
1μL each enzyme  
15μL dH<sub>2</sub>O

DNAs. 21, 38, 56, 81

Enzymes RI-H3

or

RI-SacII-H3

37° O.V.

4/16/97 new Digests (Backup)

3μL DNA (DD.2 or PrKs #LIR-Ig6)

10μL 10X RB 25mM

2μL each enzyme

83μL dH<sub>2</sub>O

37° O.V.

RI + XBA

To Page No. \_\_\_\_\_

Witnessed and

Understood by

Date

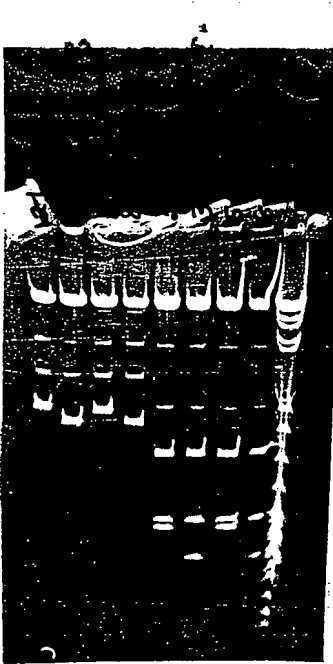
Invented by

Date

Recorded by

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4/17/97 Ran Digest on Gel.

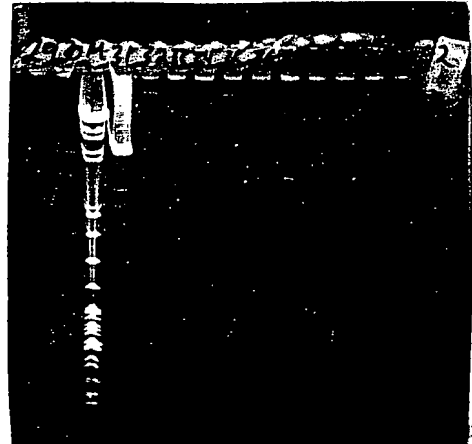
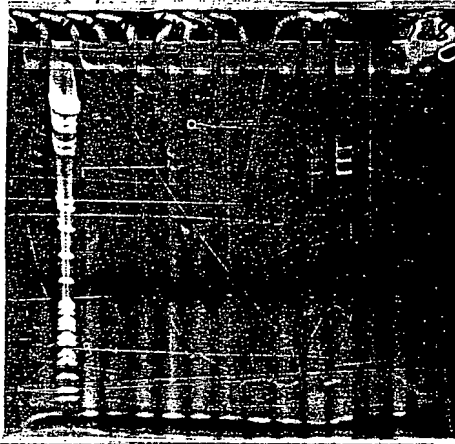
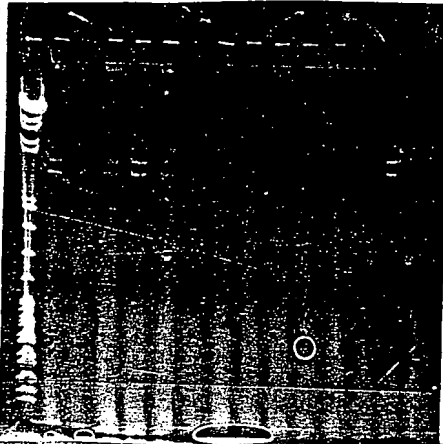


All look correct  
Gave To Mike  
For sequences.

4/17/97 set up PCRs of Transformations From 4/16  
Same protocol as on pg # 46 except.

5' primer SM.2  
3' primer UTg6 P4

Then Ran on gels



Witnessed & Understood by me,

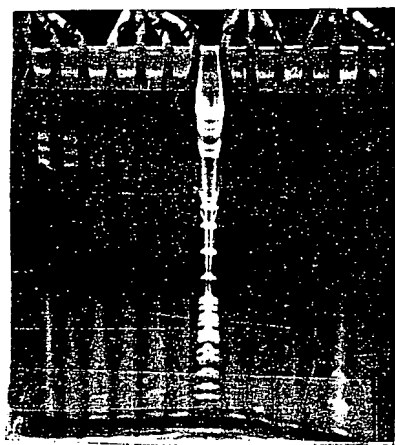
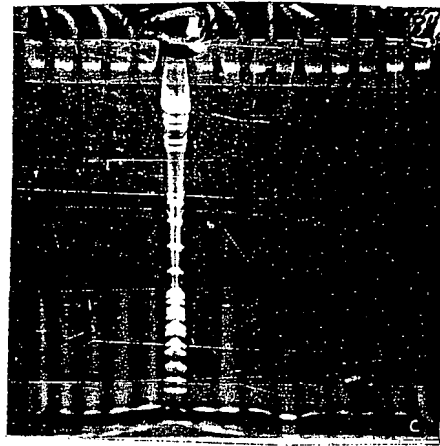
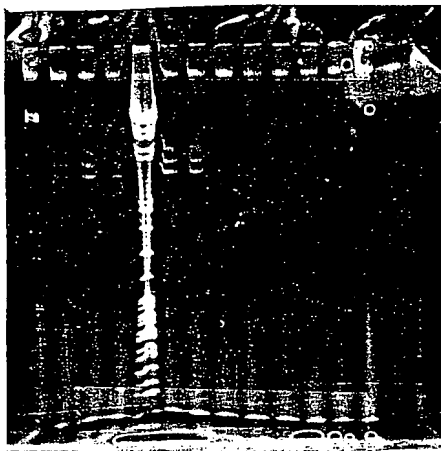
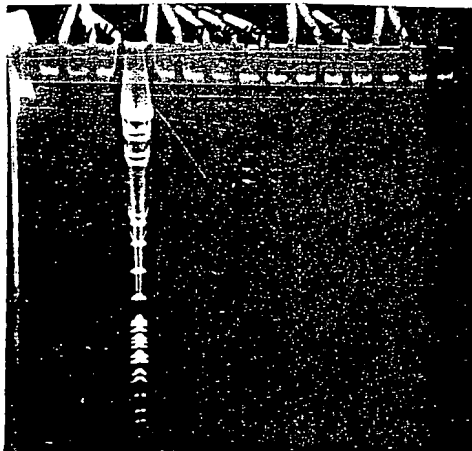
Date

Invented by

Date

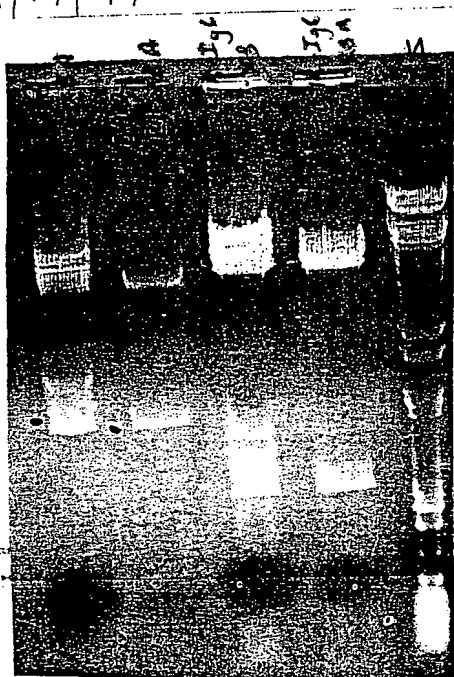
Recorded by

From Page No. \_\_\_\_\_



# 31 looks correct  
pick Template preps.  
put on wheel O.N.

4/17/97 Took Backup Digests + ran gel



cut out Bands marked.  
cleaned up with Qiaquick  
Gel extraction kit.

+ setup ligations  
Rapid Ligation kit  
SL + 1 (+ Frag)

Transformed into JM1095

To Page No. \_\_\_\_\_

With

Date

Invented by

Date

Recorded by

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4/18/97 worked up Templates on #31

Set up Mutagenesis with M1 primer +  
M2 primer.

## Kinations

1  $\mu$ L oligo  
1  $\mu$ L 10xLB  
1  $\mu$ L 10mM ATP  
1  $\mu$ L PNK  
6  $\mu$ L dH<sub>2</sub>O

37° 45 min.

1  $\mu$ L K. nasid oligo  
1  $\mu$ L Template  
1  $\mu$ L annealing Buffer  
6  $\mu$ L dH<sub>2</sub>O

5 min 68°

30 min 37°

5 min RT

Spin Down.

Add

1  $\mu$ L synthesis Buffer  
1  $\mu$ L ligase  
1  $\mu$ L T4 polymerase

5 min RT

2-4 hrs 37°

Add 90  $\mu$ L dH<sub>2</sub>O

Transformed into JM109s.

4/19/97 picked F. ITERS.

## Also

Label probe.

2  $\mu$ L oligo  
3  $\mu$ L 10xLB  
3  $\mu$ L PNK  
10  $\mu$ L  $\gamma$ P<sup>32</sup> ATP  
12  $\mu$ L dH<sub>2</sub>O

37° 45 min

Clean upon Quickspr  
column.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

From Page No. \_\_\_\_\_

4/19/97 Jamie Lyse, N<sub>out</sub>, 2xssc Filters +  
Baked 2hrs prehyb + Hybed Filters ON

4/20/97 washed Filters  
1x 6xssc RT  
2x 0.5xssc 37°  
2x 0.2xssc 37°

put up For exposure. Below.

picked colonies as mini screens.

M1 3, 6, 49  
M2 7, 17, 29

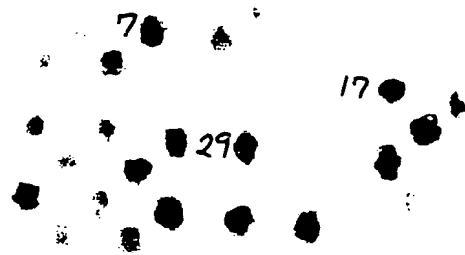
Witnessed &amp; Understood by me.

Date

Invented by

Date

Recorded by



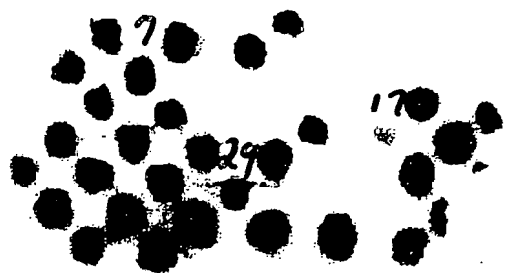
6 11 3



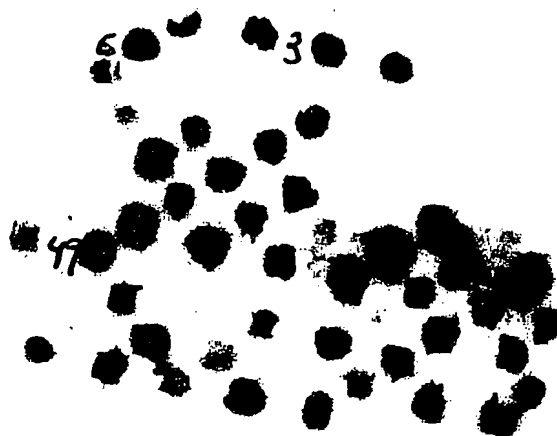
4/20/97

1hr

M2



M1



4/21/97



TITLE \_\_\_\_\_

From Page No. \_\_\_\_\_

4/21/97 M.cro screen M1 3, 6, 49  
M2 7, 17, 29

1<sup>ST</sup> Three steps of mini screen.

+ Transform into JM 109's

let Grow 0.1V.

4/22/97 pick FILTERS OF above +  
let Grow 6 hrs

Lysc Neutralize, 2xSSC, ~~in~~ A.r Dry  
Vacume Dry 2hrs + Hyb ON in  
Same probe as used on 4/19/97.

4/23/97 wash FILTERS 1x 6xSSC RT  
2x 0.25SSC 370  
+ PUT UP FOR exposure.

Witnessed & Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

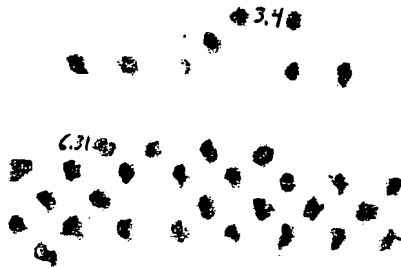
Invented by \_\_\_\_\_

Date \_\_\_\_\_

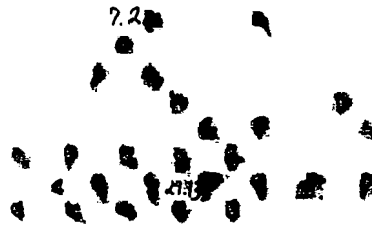
Recorded by \_\_\_\_\_

4/23/97

M1



M2



p.zk plasma  
preps of

M1

3.4  
6.31

M2

7.2  
29.43

4/23/97

M2

M1

31 2 3 4  
5 6 7 8 9  
10 11 12 13 14 15

631 32 33 34 35 36  
37 38 39 40 41 42 43 44  
45 46 47 48 49 50 51  
52 53 54 55 56 57 58 59  
60 61 62 63 64

21 2 3 4 5  
6 7 8 9 10 11  
12 13 14 15 16 17 18  
19 20 21 22 23 24  
25 26 27 28 29 30 31  
32 33 34 35 36 37 38 39  
40 41 42 43 44 45 46  
47 48 49 50 51

From Page No. \_\_\_\_\_

4/23/97

prcted plasmid preps of

M1

3.4 + 6.31

M2

7.2 + 29.43

**MICROFILMED**  
**DO NOT USE**  
**IN ORDER TO MAINTAIN**  
**DOCUMENT/FILM INTEGRITY**  
**DOCUMENT IMAGING DEPARTMENT**  
**GENENTECH**

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

**NOTEBOOK NO.** 27505  
**ISSUED TO** Scot Marsters  
**ON** 4/16 **19** 97  
**DEPARTMENT** Molecular Oncology  
**RETURNED** 19

—SCIENTIFIC NOTEBOOK CO.—  
2831 LAWRENCE AVE.  
P.O. BOX 238  
STEVENSVILLE, MI 49127  
616-429-8285

**ADE- 26**  
USSN 10/052,798

From Page No. \_\_\_\_\_

4/18/97 set up Tx in 293S cells.  
20 plates

Fed 12:00 noon

Tube A

Tube B

10mls 1:10 TE  
20  $\mu$ L VARN A (OD.2)  
200  $\mu$ L PKs Apo-2 ECD Flag  
1 ml 2.5M CaCl<sub>2</sub>

10mls 2x HBS

Mix A into B  
wait 5 min + plate 1 ml/plate.

DNA on cells 3:30 pm.

4/19/97 Fed plates serum free media.

4/21/97 Harvested 40mls to set up expt

4/22/97 Harvested rest of material + gave to  
Bob for purification.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Recorded by

From Page No. \_\_\_\_\_

4/21/97 Electro porated Helas  
set up 2 sets of each & combined to 1 plate  
10µg Plasmid / set + 1µg VARNA 60MM

Plate #	DNA.
1	PKS
2	Apo-2
3	DR4
4	Apo-3
5	TNFR1

~~DATA~~  
Electroporated  
1:00 PM.

4:00 PM Fed & washed  
cells

Added  
ZVAD. 100µM.  
(6µL OF 50MM STOCK INTO  
3 mL media.)

4/22/97 Set up NFKB Assay.  
As usual.

A's had nothing added.

B's had 5µL OF Cold Probe Added

C's had 3µL OF α NFKB AB Added. 15 min AFTER START OF RXN.

Gel loading order

Gel 1

1A/2A/3A/4A/5A/1C/2C/3C/4C/5C/

4/22/97

expose O.N.

Gel 2

1B/2B/3B/4B/5B

Witnessed & Understood by me,

Date

Invented by

Date

Recorded by

+ Cold  
01:90

$$+ \propto \sqrt{F_K B} \quad A_b.$$

4/23/97



TITLE

SAPK

Assay

Project No.

Book No. 27505

3

From Page No.

4/21/97 Electroporated HeLa's

Set up 2 sets of each + combined to 1 plate

10ug plasmid/set + 1ug VARNH

60MM

plate #

DNA

1

PKS

2

Apo2

3

DR4

4

Apo-3

5

TNFR1

Electroporated

1:00 PM

400 PM Fed + washed cells.

Added ZVAD 100uM  
(6uL OF 50M STOCK)  
into 3mls media

4/22/97 set up SAPK Assay.

To harvest cells under nondenaturing conditions: remove media and rinse cells once with ice-cold PBS.

Remove PBS and add 1.0 ml ice-cold 1X Cell Lysis Buffer plus 1 mM PMSF to one 10 cm plate and incubate the plate on ice for 5 minutes.

Scrape cells off the plate, transfer to microcentrifuge tubes. Keep on ice.

Sonicate 4 times for 5 seconds each on ice.

Microcentrifuge for 10 minutes at 4°C, and transfer the supernatant to the new tube. The supernatant is the cell lysate; if necessary lysate can be stored at -80°C.

- Microcentrifuge for 30 seconds at 4°C. Wash pellet twice with 500  $\mu$ l of 1X Lysis Buffer and twice with 500  $\mu$ l of 1X Kinase Buffer. Keep on ice.

#### all Down" SAP Kinase Using c-Jun Fusion Protein:

To 250  $\mu$ l of cell lysate add 2  $\mu$ g of c-Jun fusion protein beads. Incubate with gentle rocking overnight at 4°C.

100ug/ml use 20uL

#### Kinase Assay:

- Suspend pellet in 50  $\mu$ l 1X Kinase Buffer supplemented with 100  $\mu$ M ATP.
- Incubate 30 minutes at 30°C.
- Terminate reaction with 25  $\mu$ l 3X SDS Sample Buffer.
- Boil the sample for 5 minutes, microcentrifuge for 2 minutes.
- Load the sample (20  $\mu$ l) on SDS-PAGE gel.

1uL/10uL

Analyze sample by western blotting (see Western Immunoblotting protocol). Probe with Phospho-specific-c-Jun antibody (1:1000 dilution).

#### Western Immunoblotting:

##### Membrane Blocking & Antibody Incubations:

(volumes for 10 cm x 10 cm [100 cm<sup>2</sup>] of membrane; for different membranes sizes, adjust volumes accordingly)

- (optional) After transfer, wash membrane with 25 ml TBS for 5 minutes at room temperature.
- Incubate membrane in 25 ml of Blocking Buffer for 1-3 hours at room temperature or overnight at 4°C.
- Incubate membrane and primary antibody (at the appropriate dilution) in 10 ml Primary Antibody Dilution Buffer with gentle agitation overnight at 4°C.

use 1200 gels

phospho  
c-Jun  
1:1000  
use  
10uL.

Witnessed &amp; Understood by me,

Date

Invented by

Date

Recorded by

7

From Page No. \_\_\_\_\_

4/21/97

Tube #	use SMLs media	use SML Apo2L (1 $\mu$ g/ml Final)	IP
1	SF media		Ni Resin
2	Apo2 media		"
3	"	+ Apo2L 5 $\mu$ l (0.84 mg/ml)	"
4	SF media	"	"
5	"		"
6	Apo2 media		"
7	"	+ Apo2L 5 $\mu$ l (0.84 mg/ml)	"
8	SF media	"	"
9	SF media		$\alpha$ Flag Resin
10	Apo2 media		"
11	"	+ Apo2L 5 $\mu$ l (0.84 mg/ml)	"
12	SF media	"	"
13	"		"
14	Apo2 media		"
15	"	+ Apo2L 5 $\mu$ l (0.84 mg/ml)	"
16	SF media	"	"

Incubate media with Apo2L 30 min RT. ON ROTATOR.

Add 25  $\mu$ l of washed Resin (in TBS) 1.5 hr 4° ON ROTATOR

Wash Resin 4X TBS.

Add SB + DTT Boil 10' + Load gel.

Gel #1 M/1/2/3/4/9/10/11/12

Gel #2 13/14/15/16/5/6/7/8/M

+ Blot to Nitrocellulose

put in 5% milk to block  
O.N.To Page No. 6

Witnessed &amp; Understood by me,

Date

Invented by

Date

Recorded by

2935 cells For TX.

TITLE \_\_\_\_\_

Project No. \_\_\_\_\_  
Book No. 27505

5

From Page No. \_\_\_\_\_

2935 100mm Dishes.

4/21/97 Set up 160 plates For TX Later in The week.

4/25/97 Fed cells 12:00 noon.

100 plate TX with Apo-2-Ig6

60 plate TX with Apo-2-EC0-Flag.

+ 1ug VARNNA / 10ug DNA / plate

DNA on cells 4:00 pm

4/26/97 Fed cells Serum Free media

4/28/97 <sup>am.</sup> Took samples & Gave to Assay services  
For FC elisa. pg# 8.

4/28/97 <sup>p.m.</sup>

Harvested S.F. sup of Ig6 plates  
& Gave to Bob to purify.

Re Fed plates with new S.F. media

5/2/97 Harvested Sups From plates.

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

Recorded by

From Page No. 4

4/22/97 wash Blots 1X TBST

Gel 1 3 $\mu$ L  $\alpha$  Flag (M2)Gel 2 2 $\mu$ L polyclonal #3  $\alpha$  Apo2L

in 10 mLs.

TBST +

5% BSA.

RT 2 hrs.

wash 3X TBST.

Gel 1 10 $\mu$ L Sheep  $\alpha$  Mouse IgG HRPGel 2 5 $\mu$ L  $\alpha$  Rabbit HRP

in 10 mLs

TBST +

5% M.I.K.

wash 3X TBST

expose using LumiGlo (NEB)

Develop Films.

4/22/97

WB: αFlag

IP: Ni

αFlag

SPN 2 2H 2° AP 2 2H L



IP:

Ni

αFlag

213  
107  
69  
45  
28.6  
18.7  
15.2

Apo-2L  
Apo-2+L  
Apo-2  
SPN  
Apo-2L  
Apo-2L  
Apo-2  
SPN

WB: αApo-2L

4/22/97

WB  $\alpha$  Flag

IP

Ni

$\alpha$  Flag

SFM 2 24L L

SFM 2 24L L



IP

Ni

$\alpha$  FLAG

213

167

69

45

28.6

18.7

15.2

Apo2L

Apo2+L

Apo2

SFM

Apo2L

Apo2L

Apo2

SFM

WB:  $\alpha$  Apo2L

IP

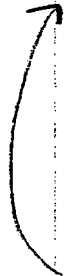
SF  
Apo2  
Apo2 + Apo2L NI  
Apo2L -

SF  
Apo2  
Apo2 + Apo2L Flag  
Apo2L

SF  
Apo2  
Apo2 + Apo2L NI  
Apo2L -

SF  
Apo2  
Apo2 + Apo2L Flag  
Apo2L

what to expect.



Apo2L

Apo2L

5mls  
1mg/ml of Apo2L

3mg/ml of Apo2L  
1mg/ml of Apo2L

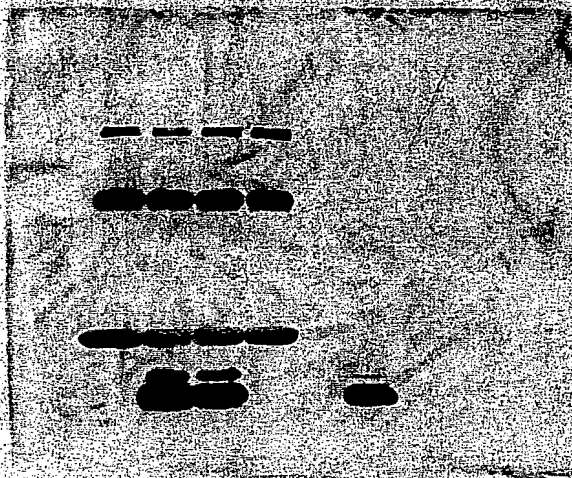
Tube #

		IP		WB
1	SF media	Ni		Flag
2	Apo-2	"	Gel 1	"
3	Apo-2 + Apo2L	"	1	"
4	Apo2L	"	(1-4)	"
5	SF media	"		$\alpha$ Apo2L
6	Apo-2	"	Gel 2	"
7	Apo-2 + Apo2L	"	2	"
8	Apo2L	"	(5-8)	"

		Flag		Flag
9	SF media	"		"
10	Apo-2	"	Gel 1	"
11	Apo-2 + Apo2L	"	1	"
12	Apo-2L	"	(5-8)	"
13	SF media	"		$\alpha$ Apo2L
14	Apo-2	"	Gel 2	"
15	Apo-2 + Apo-2L	"	2	"
16	Apo-2L	"	(1-4)	"

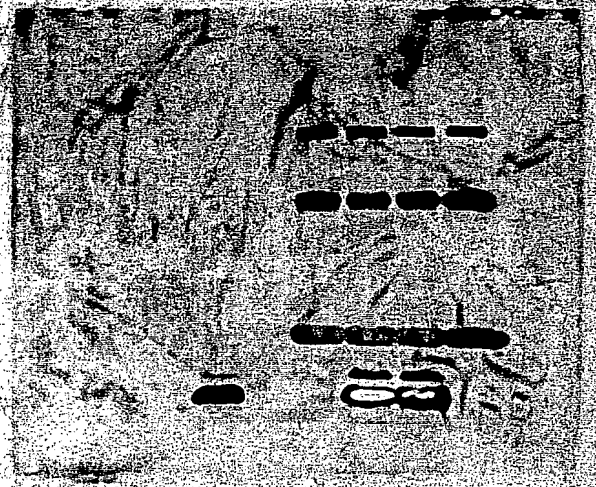


STRATA

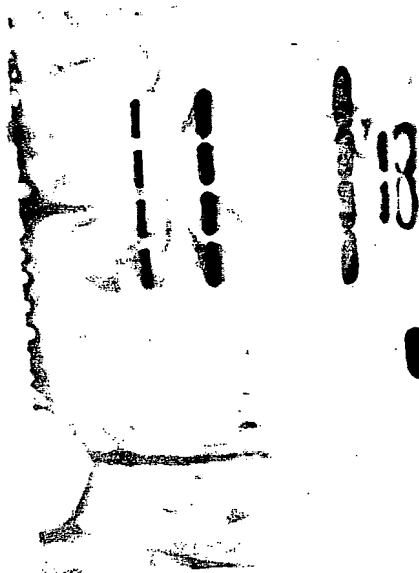


— —

4/22/97



4/22/97



KODAK SAFETY FILM A80

KODAK SAFETY FILM A80

4/22/97

11

18

18

1

1

4/22/97

4/22/97



From Page No. \_\_\_\_\_

5/6/97 set up PCR Amplification of  
cDNA

6 sets of cells

## 2.0 PCR Protocol

2.1 For each sample prepare a minimum of 78  $\mu$ L of PCR Master Mix as shown below. (See Sections 4.1 and 4.3).

Component	Volume	Final Concentration
25 mM MgCl <sub>2</sub> Solution	4 $\mu$ L	2 mM
10X PCR Buffer II	8 $\mu$ L	1X
dH <sub>2</sub> O	65.5 $\mu$ L	—
AmpliTaq <sup>®</sup> DNA Polymerase	0.5 $\mu$ L	2.5 U/100 $\mu$ L
Total PCR Master Mix volume per sample	78 $\mu$ L	—

2.2 Dispense 78  $\mu$ L of the PCR Master Mix into each reverse transcription reaction tube. Change tips between additions to avoid sample carryover.2.3 Dispense the primers into each tube. Change tips between primer additions to avoid sample carryover. For the pAW108 RNA supplied with this kit, use the DM151/DM152 primer set. If a "downstream" primer was used for the reverse transcription reaction (e.g., DM152), then it should not be added again. Instead, substitute 1  $\mu$ L dH<sub>2</sub>O.

Component	Volume	Final Concentration
DM152 "Downstream" Primer	1 $\mu$ L	0.15 $\mu$ M
DM151 "Upstream" Primer	1 $\mu$ L	0.15 $\mu$ M

The final volume of each tube should now be 100  $\mu$ L (including 20  $\mu$ L from the reverse transcription reaction).

2.4 Spin the tubes for approximately 30 seconds in a microcentrifuge.

3.0 Temperature Cycling for the Control Reagents. Optimum performance of the GeneAmp<sup>®</sup> PCR Process is achieved using a GeneAmp<sup>®</sup> PCR Instrument System.Store samples at 8°C or less until ready to use (with the GeneAmp<sup>®</sup> PCR Carry-over Prevention Kit [Part No. H808-0086] use 72°C).

Table 3. Perkin-Elmer GeneAmp PCR Instrument Systems' PCR Profile Times and Temperatures.

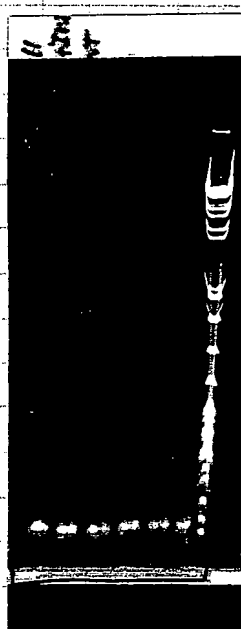
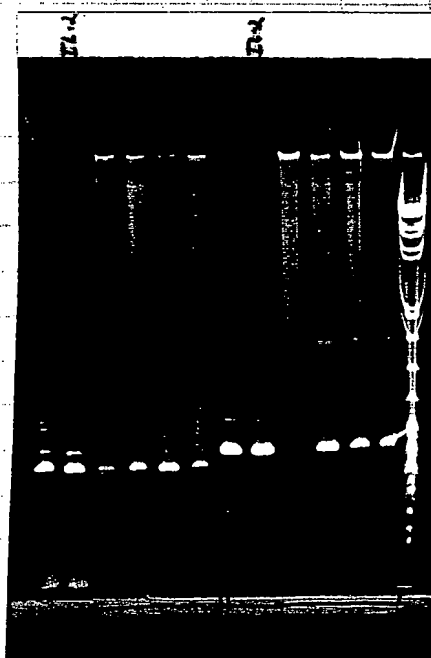
Perkin-Elmer GeneAmp <sup>®</sup> PCR Instrument System	Tube (for PCR)		Times & Temperatures for Amplification with this Kit			
	Tube Type	Volume ( $\mu$ L/tube (approx. 100 $\mu$ L))	Initial Step	Each of 35 Cycles		Final Step
				Melt	Anneal-Extend	
DNA Thermal Cycler or DNA Thermal Cycler 480	GeneAmp PCR Reaction (9601-0189)	100 (40-100 $\mu$ L)	STEP CYCLE	STEP CYCLE	STEP CYCLE	TIME DELAY
			120 sec. 95°C 1 cycle	80 sec. 95°C	80 sec. 60°C	7 min. 72°C
DNA Thermal Cycler 480	GeneAmp Thin-Walled Reaction (9601-0207) (9601-0211) (9601-0227)	100 (40-100 $\mu$ L)	STEP CYCLE	STEP CYCLE	STEP CYCLE	TIME DELAY
			120 sec. 95°C 1 cycle	45 sec. 95°C	45 sec. 60°C	7 min. 72°C
GeneAmp <sup>®</sup> PCR System 9600 (System 2400)	MicroAmp <sup>®</sup> Reaction (9601-0329) (9601-0349) (9601-0372)	100 (no vapor barrier needed)	HOLD	CYCLE CYCLE		HOLD
			105 sec. 95°C	15 sec. 95°C	30 sec. 60°C	7 min. 72°C

Tcells  
Tcells + IL-2  
Jurkat  
Hela  
293S  
9D

Sets of Primers For

- 1) Apo 2  $\approx$  170 Bp Frag
- 2) DR 4  $\approx$  200 Bp Frag
- 3) Actin  $\approx$  100 Bp Frag

5/7/97



To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

NOTEBOOK NO. 26577  
ISSUED TO Maya Skubatch  
ON [REDACTED] 19 [REDACTED]  
DEPARTMENT Molecular Oncology  
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ADE-27  
USSN 10/052,798



From Page No. \_\_\_\_\_

Pet HuAP02L  
Pick #23 for plasmid prep.

Spleen newL.2

Wash

1X 6XSSC R.T.  
2X 0.5XSSC  
1% SDS 37°  
Expose O.N.

DD.2 Receptor  
Purify (RI-H3) fragment using Qiagen Agarose technique

DD.2 DNA  
80ng/ml



Label with probe

① 3 ul DD.2 (RI-H3)  
2 ul dH<sub>2</sub>O  
10 ul primers (brn cap)  
100° 5'

② 10 ul dCPT Buffer (blk cap)  
1 ul Klenow (grn cap)  
5 ul dCPT α  
37° 15'

on column 25K  
50 ul ss Salmon Sperm  
5' 100K  
add to hybridization

Witnessed &amp; Understood by me,

Date

Invented by

Date

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TITLE \_\_\_\_\_

From Page No. \_\_\_\_\_

maxi prep

2L - DD.2

2L - NGFRrp #2

Blots mRNA

Strip blots:

boil 1L dH<sub>2</sub>O in 2L flask w/ 5ml 20% SDS.

dry blots on paper, air dry.

use expresshyb soln' (at 65°) and hyb blots 30'

transfer blots to new plate w/ express hyb and add hot probe.

(see p. 76)

Hyb O.N. 65°

USER: 1	ID: 32P	COMMENTS: 32P	
PRESET TIME: 2.00	H#: NO	SAMPLE REPEATS: 1	
PRINTER: STD	SCR: NO	REPLICATES: 1	
RS232: OFF	RCM: YES	MULTIPLIER: 1.0000	

ISOTOPE 1: 32P %ERROR: 0.00 BKG. SUB: 0

SAM	POS	TIME	32P		RCM	E'
NO		MIN	CPM	%ERROR		

1	1-1	2.00	97819.54	0.45	0.01	
---	-----	------	----------	------	------	--

∴ 50,000,000 cpm / 50 ml

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Recorded by

Date

From Page No. \_\_\_\_\_

## Northern Blots Wash

## Wash Soln' 1

2X SSC (100 mls/L)  
0.05% SDS (2.5 mls/L)

## Wash Soln' 2

0.1X SSC (5 mls/L)  
0.1% SDS (5 mls/L)

- Rinse blot in soln 1 Several times R.T for 30-40 minutes
- Wash blot in soln 2 2x shaking continuously at 50°C
- expose to phosphorimager

## Spin down HuAPO2L-pet

## Maxiprep

## HuAPO2L-pet19b

DD.2 from Spleen Library.

## APO2L - Mutagenesis

pick 100 colonies of P, PI and plate &amp; master plate

O.N. 87 → 10-53 probe

make probe: 2ul DNA, 3ul 10xUB, 3ul PNK, 1ul ATP, 12ul dH<sub>2</sub>O 37° 45', Purify on column

Develop Spleen new L.2 → Very wk signal / probe not specific

Pick 4 wk plugs elute 1/2 hr 37° 0.5ml PSP ChCl<sub>3</sub>

PCR see p. 73

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

TITLE \_\_\_\_\_

From Page No. \_\_\_\_\_

Kidney.  
 Liver  
 Lung  
 Brain

PBL  
 Colon  
 sm intestine  
 Ovary  
 Testis  
 prostate  
 Thymus  
 Spleen

pancreas  
 Kidney  
 SK. Muscle  
 Liver  
 Lung  
 Placenta  
 Brain  
 Heart

Northern  
 1hr  
 exposure

Witnessed & Understood by me,

Date

Initiated by

Recorded by

Date

*Mary Skubitch*



From Page No. \_\_\_\_\_

PCR X100 94° 3' 1 cycle  
 10 ul dH<sub>2</sub>O + colony —  
 12 ul dNTP's 1200 ml 94° 1' 25 cycles  
 5 ul 10xPCR Buffer 500 ul 50° 1'  
 0.5 ul IgG.P.4 50 ul 72° 3'  
 0.5 ul sm. 2 50 ul  
 32 ul dH<sub>2</sub>O 32 ml  
 0.5 ul Taq Polymerase 50 ul

#	Sample
1-18	1A
19-36	1C
37-54	1E
55-56	—
57-60	2A
61-	2C
62-72	2E
73-74	3A
75-81	3E
82-96	1E

AP02L

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

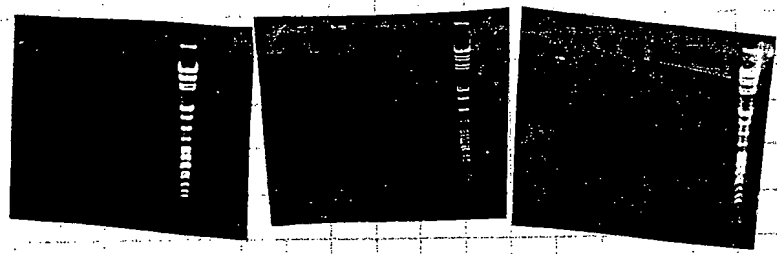
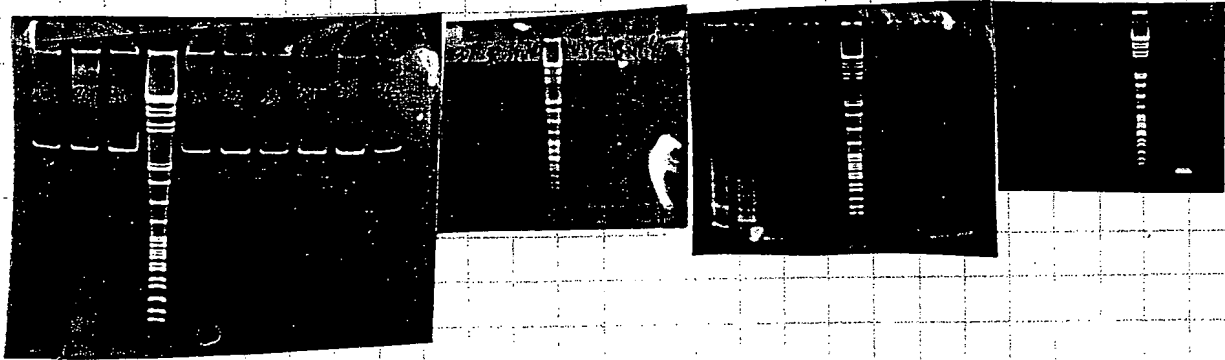
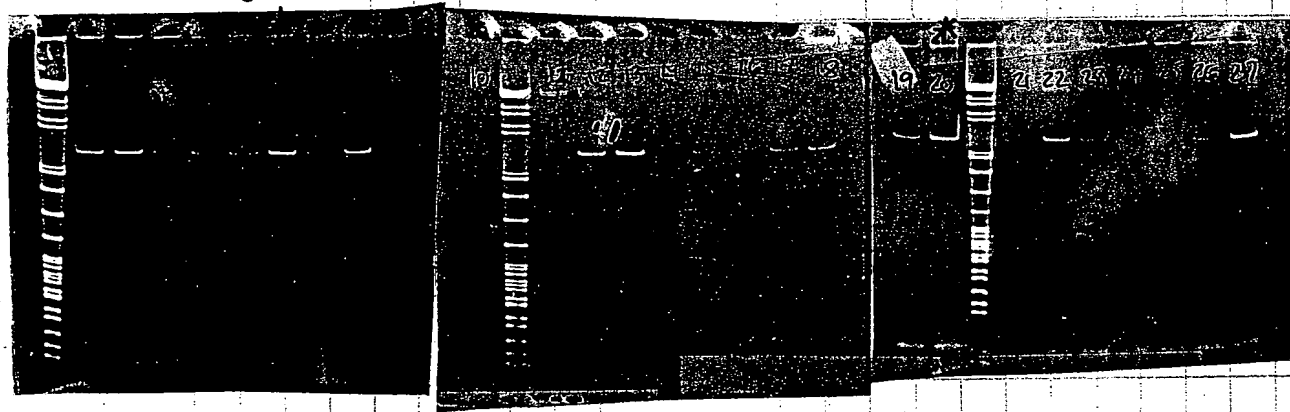
Invented by

Date

Recorded by

From Page No. \_\_\_\_\_

DD-2-IgG



Pick G, 20 for IL Prep

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by *[Signature]*  
Recorded by *[Signature]*

Date

From Page No. \_\_\_\_\_

AP02L-mut

pick master plate & filter of AP02Lmut Rd 2

#1-25 P "4"

#26-50 P "81"

#51-75 P 31

#76-100 P 62

DD.2-IgG

Maxi Prep #6, #20

AP02L mutagenesis Rd 2

type, Nutt, air dry. vac dry 1hr 80°, pre hyb, hyb, O.N.

Spleen

Her library

DD.2/ Radiation Hybrid

2.5 ul DNA

2.5 ul PCR Buffer (Clontech)

6.0 ul dNTP'S

0.35 ul D1 (00-PCR 5')

0.5 ul P2 (DD-PCR 3')

0.25 ul T4 Polymerase

13 ul dH<sub>2</sub>O

25 ul Total

X100

250

600

25

25

1300

94° 3' 1 cycles

94° 1' 60° 1' 72° 2' 35 cycles

1-78, 81-85

84 G3/RM +

85 G3/A3 -

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by W. Jamblich

Recorded by

Date



From daemon

Date:

From: rhserver@toolik.Stanford.EDU (Radiation Hybrid Mapping Email Server at SHG C)

To: sam@gene.COM

Subject: rhserver@shqc: completed two point calculations

This email message has been sent automatically by  
rhserver@shgc.stanford.edu in response to your submission:

messageID: [REDACTED] OAA25204.

reference: dd.2

The following information contains the SHGC framework marker which best links with your marker with a LOD score of 6 or greater. Note that if you want to consider LOD scores lower than 6, you must provide a chromosomal assignment for your marker.

The results of this query are designed to be used in conjunction with the G3 maps found on the SHGC web page. Please visit our RHPMapping section at <http://www-shgc.stanford.edu/rh/frames/engine.html> and query with the name of the linked marker for information about map position and other markers in the area.

For reference, your original email submission is appended to these results.

Disclaimer: Neither the Stanford Human Genome Center nor Stanford University make any claims about veracity or suitability of these results. This information is provided on an as-is basis only.

Two Point Maximum Likelihood Analysis results for submitted marker dd.2.

The raw scoring data was:

[illegible]

The calculation results were:

submitted marker	linked marker	LOD	cR_10000	chrom
------------------	---------------	-----	----------	-------

Reporting best lod  $\geq 6.0$ 

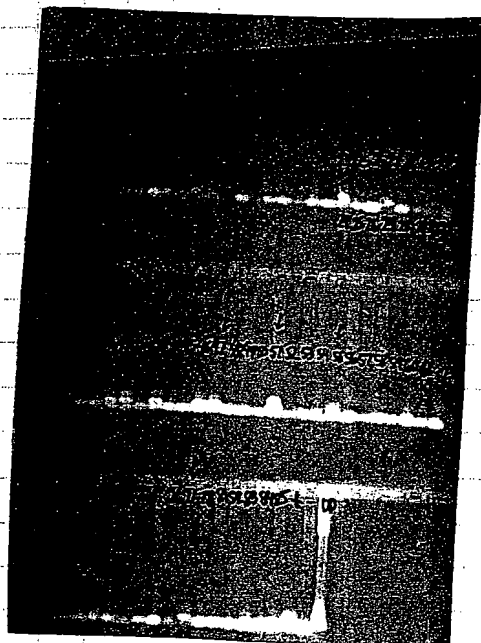
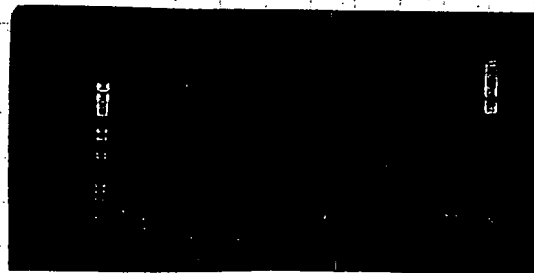
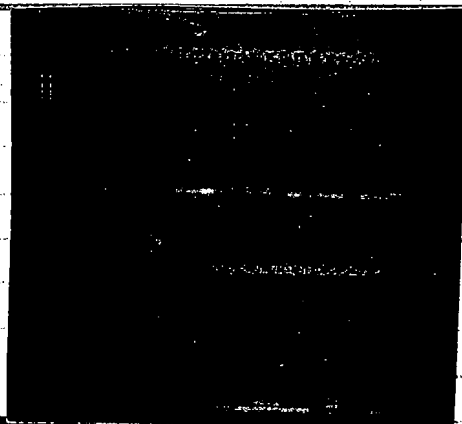
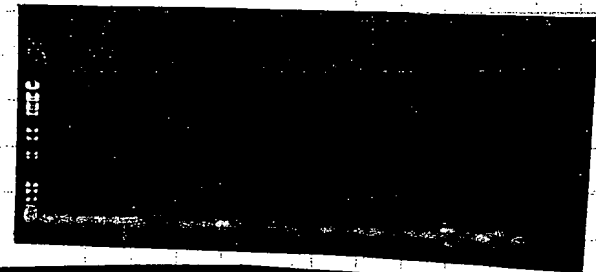
dd.2	D8S481	11.054567	9.90	8
------	--------	-----------	------	---

Original submission:

>From nobody

From Page No. \_\_\_\_\_

→ Run Hybrid PCR on gel



AP02L-Mutagen Rel 2

Wash 1x 6XSSC R.T.  
 2x 0.2XSSC  
 1% SDS 37°

2x 0.1XSSC  
 1% SDS 42°

Spleen library / aliquot w/ Green Cap 1139a 2gto plate 10<sup>-4</sup> 10ml

To Page No. \_\_\_\_\_

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Date

Invented by

Date

Recorded by

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March 17, 1997 - Monday

Plate 20X  $10^{-4}$  spleen agtio library

PCR Prk5 DO Race

Sample	Library
1-6	Hu fetal lung > 2500
7-12	Hu fetal lung 1.3-2.5
13-18	Hu fetal lung 0.6-1.3
19-24	Hu fetal small intes > 2500
25-30	Hu fetal small intes 1.3-2.5
31-36	Hu fetal small intes 0.6-1.3
37-42	fetal kidney prk5b < 2.8 kb
43-48	fetal lung < 2.8 kb
49-54	fetal kidney prk5 > 2.8 kb
55-60	fetal lung > 2.8 kb
61-66	fetal liver prk5 1:66

1  $\mu$ l cDNA library  
12  $\mu$ l dNTPs  
5  $\mu$ l PCR buffer  
0.5  $\mu$ l P1  
0.5  $\mu$ l P2  
31  $\mu$ l dH<sub>2</sub>O

Primer sets

A	prk Race 1	+	DD Race 3
B	prk Race 1		DD Race 2
C	SM 1	+	DD Race 3
D	SM 1	+	DD Race 2
E	prk Race 2		DD Race 3
F	prk Race 2		DD Race 2

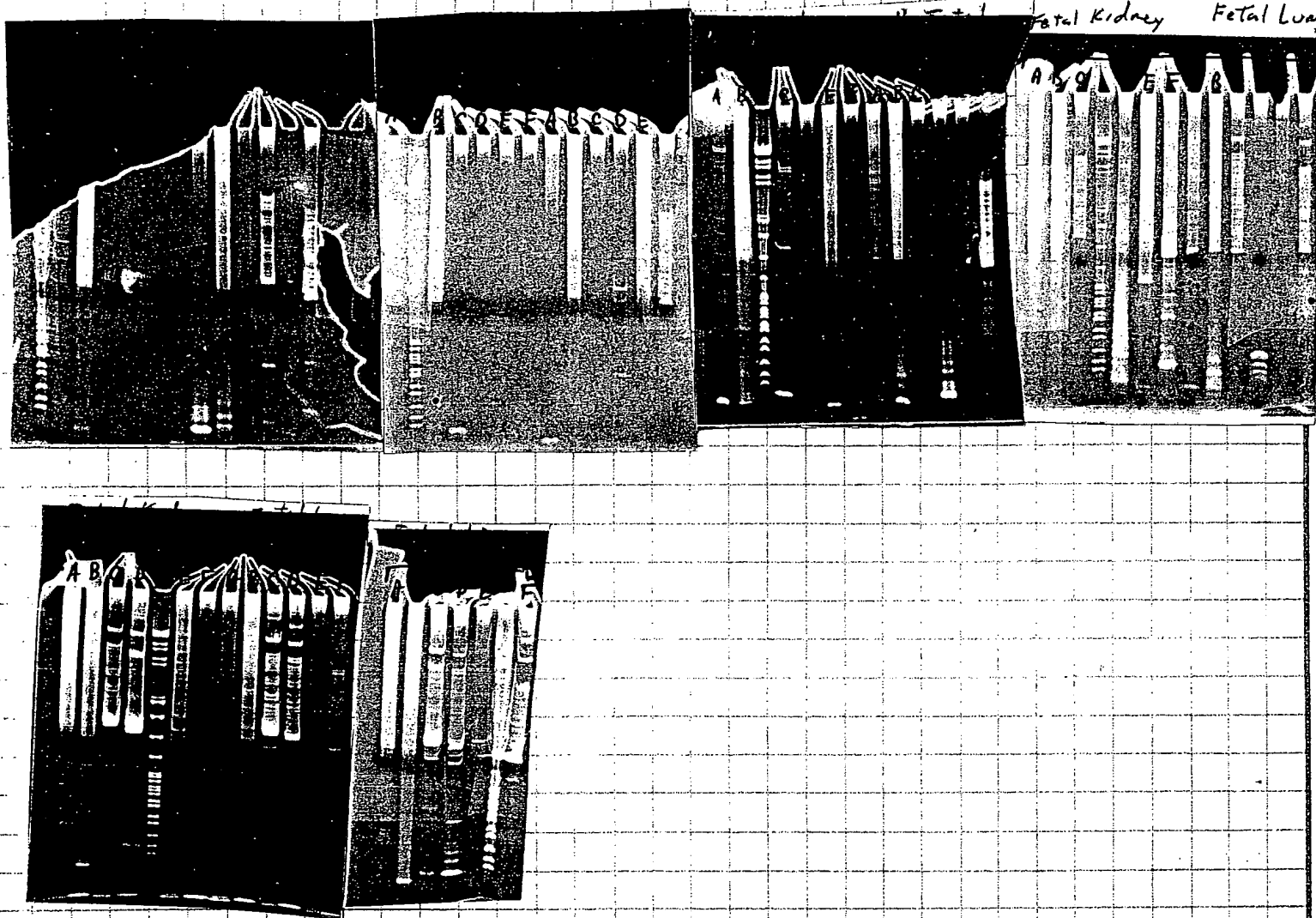
94° 1' 60° 1' 72° 2' 35 cycles

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		Recorded by	

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Pick APO2.L mut #10, 16, 82 → 500ml for sequencing



March 18, 1997 - Tuesday

Re-PCR 1ul aliquot (2<sup>nd</sup> Round)

#10	1.	#10	(SM.2 DD. race 1) Hf si 1.3-2.5	6	#39	(SM.2 DD. race 2) f k < 2.8
	2.	#22	(SM.2 DD. race 1) Hf si 1.3-2.5	7	#39	(SM.2 DD. race 1) f k < 2.8
	3.	#27	(SM.2 DD. race 2) Hf si 1.3-2.5	8	#44	(prk. race 2 DD. race 1) f l < 2.8
	4.	#27	(SM.2 DD. race 1) Hf si 1.3-2.5	9	#49	(prk. race 2 DD. race 2) f k > 2.8
	5.	#38	(prk. race 2 DD. race 1) f k < 2.8	10	#49	(prk. race 2 DD. race 2) f k > 2.8

Page No. Cont

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Date \_\_\_\_\_

Inverted by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

*Skubatch*

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#11	#51	(SM.2	OD race.2)	fk	>2.8	<del>1ul</del>
12	#51	(SM.2	OD race.1)	fk	>2.8	
13	#58	(SM.2	OD race.1)	fl		
14	#62	(Prk race.2	OD race.1)	fl		
15	#63	(SM.2	OD race.2)	fl		
16	#63	(SM.2	OD race.1)	fl		

PCR Reaction

X 200

2ul DNA

1 ul P1

1 ul P2

24 ul 1.25mm dNTP

10 ul PCR buffer

1 ul Taq

61 ul dH<sub>2</sub>O

480

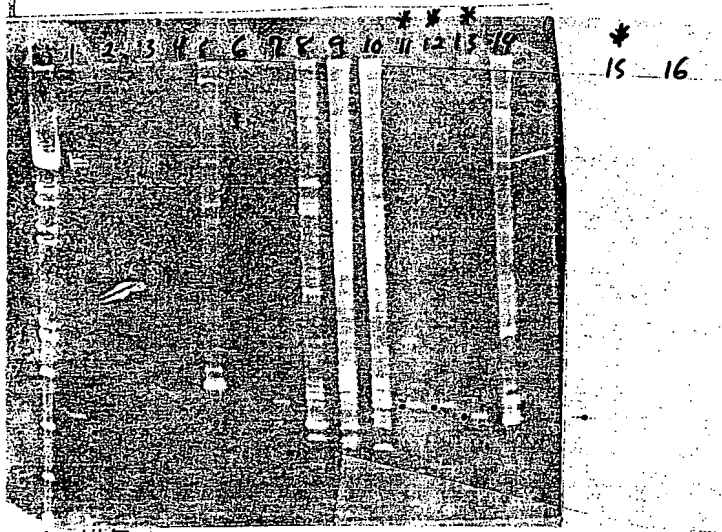
200

20

1220

94° 1' 60° 1' 72° 2'

16 cycles



10 G3

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Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

TITLE \_\_\_\_\_

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PCR  
redo

		Library	Size	Primers
1	#10	Hu fetal lung	1.3-2.5	SM1, DD Race 2
2	22	Hu fe small intes	725	SM1, DD Race 2
3	27	Hu fe small intes	1.3-2.5	SM1, DD Race 3
4	39	fetal kidney	<2.8	SM1, DD Race 3
5	51	fetal kidney	<2.8	SM1, DD Race 3
6	58	fetal lung	728	SM1, DD Race 2
7	63	fetal liver		SM1, DD Race 3

PCR rxn

x10

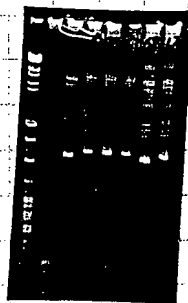
1 ul cDNA  
 1 ul P1  
 1 ul P2  
 10 ul PCR Buffer  
 24 ul dNTP's  
 1 ul Tag  
 62 ul dH<sub>2</sub>O

100

240

10

620



Maxi Prep APO 2C - mutagenesis 10, 82, 76

Spleen

Pull Filters

Lyse

Need

air dry.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Recorded by \_\_\_\_\_

Date \_\_\_\_\_

From Page No. \_\_\_\_\_

Wednesday 3/19/97Run PCR on 6% TBE gel p91Probe screen: 3ul DNA, 3ul 10xUB, 3ul PNE, 10ul ATP<sup>32</sup>(8), 12ul dH<sub>2</sub>O 45' 37"  
Spn on column / Probe new 6.1 O.N/R.TPurify fragments (11, 12, 13, 15) (2-7)

## Technical Bulletin

Promega

## IV. Sample Preparation

Note: Thoroughly mix the PCR Preps DNA Purification Resin before removing an aliquot. If crystals or aggregates are present, dissolve by warming the resin to 25-37°C for 10 minutes. The resin itself is insoluble. Cool to 30°C before use.

## A. Purification of DNA from Agarose

Purify PCR products using agarose gel electrophoresis if nonspecific amplification products must be removed.

1. Separate the PCR reaction products by electrophoresis in a TAE agarose gel containing ethidium bromide using standard protocols (2).
- Note: We do not recommend the use of TBE-containing gels with Wizard™ PCR Preps.
2. Excise the desired DNA band using a clean, sterile razor blade or scalpel.
- Note: The band should be visualized with a medium or long wavelength (e.g., 230nm) UV light, and should be excised quickly to minimize exposure of the DNA to UV light. The band should be isolated in approximately 300µl (300mg) of agarose. For isolation from larger agarose slices, see reference 3.
3. For high-melting temperature agarose, use Step 3.a. For low-melting temperature agarose, use Step 3.b.
  - a. High-melting temperature agarose: Transfer the 300µl (300mg) agarose slice to a 1.5ml microcentrifuge or screw cap tube and add 1ml of resin. Incubate at approximately 65°C in a waterbath for 5 minutes or until the agarose is completely melted. Proceed directly to Step 5.
  - b. Low-melting temperature agarose: Transfer the 300µl (300mg) agarose slice to a 1.5ml microcentrifuge tube and incubate the sample at 70°C until the agarose is completely melted. Proceed directly to Step 4.
4. Add 1ml of resin to the melted agarose slice. Mix thoroughly for 20 seconds but do not vortex.
5. If you plan on using a vacuum manifold, proceed to Section V. Otherwise, proceed to Section VI.

## B. Sample Preparation for Direct Purification from PCR Reactions

1. For each completed PCR reaction, transfer the aqueous (lower) phase to a clean microcentrifuge tube. The presence of too much mineral oil in the sample can lead to a decreased yield in the PCR product purification.
2. Aliquot 100µl of Direct Purification Buffer into a 12 x 75mm polypropylene tube or a 1.5ml microcentrifuge tube. Add 30-300µl of the PCR reaction. Vortex briefly to mix.

Revised 5/96

## Technical Bulletin

Promega

3. Add 1ml of resin and vortex briefly 3 times over a one minute period.

4. If you plan on using a vacuum manifold, proceed to Section V. Otherwise, proceed to Section VI.

## V. PCR Product Purification Using a Vacuum Manifold

## A. Procedure

Reagents to Be Supplied by the User

- 80% isopropanol (2-propanol, reagent grade)
- dH<sub>2</sub>O or TE buffer

Multiple Wizard™ PCR Preps can be easily processed simultaneously with Promega's Vac-Man® Laboratory Vacuum Manifold (Cat.# A7231).

Note: Work quickly when purifying from low melting temperature agarose since re-gelling of this material will decrease yields.

1. For each PCR product, prepare one Wizard™ Minicolumn.
2. Attach the provided Syringe Barrel to the Luer-Lok® extension of each Minicolumn. Insert the tip of the Minicolumn/Syringe Barrel assembly into the vacuum manifold.
3. Pipet the resin/DNA mix into the Syringe Barrel. Apply a vacuum to draw the resin/DNA mix into the Minicolumn. Break the vacuum to the Minicolumn.
4. To wash the column, add 2ml of 80% isopropanol to the Syringe Barrel, and re-apply a vacuum to draw the solution through the Minicolumn.
5. Dry the resin by continuing to draw a vacuum for 30 seconds after the solution has been pulled through the column. Do not dry the resin for more than 30 seconds. Remove the Syringe Barrel and transfer the Minicolumn to a 1.5ml microcentrifuge tube.
6. Transfer the Minicolumn to a new microcentrifuge tube. Apply 50µl of water or TE buffer to the Minicolumn and wait 1 minute (the DNA will remain intact on the Minicolumn for up to 30 minutes). Centrifuge the Minicolumn for 20 seconds at 10,000 x g to elute the bound DNA fragment.

Note: Large DNA fragments require elution at an elevated temperature with water or TE buffer that has been heated just prior to elution. Elute fragments >3kb at 65-80°C. Elute fragments >20kb at 80°C.

7. Remove and discard the Minicolumn. The purified DNA may be stored in the microcentrifuge tube at 4°C or -20°C.

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Witnessed &amp; Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

From Page No. \_\_\_\_\_

Quick Ligation of PCR into pGEMT

1 ul vector  
 7 ul insert  
 2 ul vial 2  
 10 ul vial 1  
 mix  
 1 ul vial 3  
 5 min RT

SL  
 1A #22 (Sm.1 race 2)  
 2A #27 (Sm.1 race 3) 6A #63 (Sm.1 race 3)  
 3A #34 (Sm.1 race 3) 7A #51 (Sm.2 race 3)  
 4A #51 (Sm.1 race 3) 8A #51 (Sm.2 race 1)  
 5A #38 (Sm.1 race 1) 9A #58 (Sm.2 race 1)  
 6A #63 (Sm.2 race 2)

Transformation

20' 00"  
 45" 42"  
 2' 00"

45' 37" on wheel plate 100ul ON (NZYDI / IPTG)

pet muAPO2L, pet muAPO2L w/ pet 19f/r

Submit for sequencing APO2L 10, 76, 82 w/ pcr for  
 DD.2 19f # w/ 1964

DD.2 - 5'  
 PCR

LibraryPrimer

Library	Primer	Rxn	X6
1 Human spleen	(SM.1 DD Race 3)	1 ul DNA	
2 "	( " DD Race 2)	1 ul PI	6 ul
3 Hu Thymus	( " DD Race 3)	1 ul P2	6 ul
4 "	( " DD Race 2)	10 ul Buffer	60 ul
5 Hu Genomic	( " DD Race 3)	24 ul 1.25 dNTPS	144 ul
6 "	( " DD Race 2)	1 ul Tag	6 ul
7 Hu Lung	( " DD Race 3)	62 ul dH <sub>2</sub> O	372
8 "	( " DD Race 2)		
9 Hu Liver	( " DD Race 3)		
10 "	( " DD Race 2)		
11 fe Heart	( " DD Race 3)		
12 "	( " DD Race 2)		

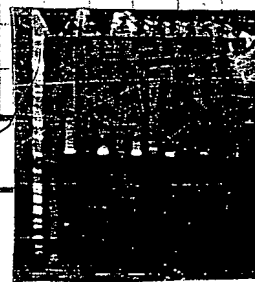
94° 1' 60° 1' 72° 2' Expts

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Date \_\_\_\_\_

Invented by \_\_\_\_\_

Recorded by \_\_\_\_\_





TITLE \_\_\_\_\_

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Friday 3.21.97

Transform 3 plar products p. 93 Hu Thymus, Lung, Spleen  
using rapid ligation into pGEM-T

1 ul vector

1 ul insert

2 ul vial 2

10 ul vial 1

Mix

1 ul vial 3

5 min RT

add 100 ul JM109

20° 0°, 45° 45°, 2' 0° 45°

add 300 ul LB

Spin 37°

plate

---

pick #10 for Maxi prep

---

To Page No. \_\_\_\_\_

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Date

Invented by

Recorded by

Date

NOTEBOOK NO. 27236  
ISSUED TO Maya Skubatch  
ON [REDACTED] 19  
DEPARTMENT Molecular Oncology  
RETURNED Jo 19

—SCIENTIFIC NOTEBOOK CO.—  
2831 LAWRENCE AVE.  
P.O. BOX 238  
STEVENSVILLE, MI 49127  
616-429-8285

**ADE- 28**  
USSN 10/052,798

From Page No. \_\_\_\_\_

Monday 3/24/97

Work up #10 (Hu spleen (sm.1, DD race.3))

Reprobe filters of spleen library with newL.2 probe.

3  $\mu$ l probe DNA3  $\mu$ l 10xLB3  $\mu$ l PNK10  $\mu$ l ATP<sup>32</sup>P12  $\mu$ l dH<sub>2</sub>O

USER: 8 ID:32P

PRESET TIME: 1.00

SAMPLE REPEAT: 1 CYCLE REPEAT: 1 SCR:N RS232:N

H#: 0 AQC:N QCF:N RCM:N

CHANNEL 1-LL: 0 UL:1000 2SIGMA: 2.00 BKG SUB: 0.

DATA CALC: CPM, UNKNOWN REPLICATES: 1 NORM FACTOR

HALF LIFE(DAYS):N

SAM	POS	CH	CPM	2SIG%	TIME	EL TIME
1	**	1	1	863840.00	0.96	0.05
						0.23

45' 37° spin on column

5' of DD.2

Religate into pGEMT and transform

1  $\mu$ l pGEMT1  $\mu$ l ~~vector~~ insert10  $\mu$ l vial one quick ligation kit2  $\mu$ l vial two " " "

Mix

1  $\mu$ l vial three

R.T. 5'

add 100  $\mu$ l JM109 Promega

20' 0° 45" 42° 2' 0°

add 300  $\mu$ l LB spin 45' 37°

plate on (LB+Carb + IPTG - X-GAL) O.N. 37°

SLA - pGEM-T

1A Hu Thymus

2A Hu Lung

3A Hu Spleen

To Page No. \_\_\_\_\_

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Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

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DD2.2 - IgG Fusion

## PCR

0.5  $\mu$ l DD2.1 DNA  
1.0  $\mu$ l SM.1 or SM.2  
1.0  $\mu$ l SM.4  
10  $\mu$ l 10x PCR Buffer  
24  $\mu$ l 1.25 mM dNTP's  
1.0  $\mu$ l Tag  
63  $\mu$ l  $H_2O$

rxn 1: SM.1

rxn 2: SM.2

94° 1' 70° 1' 72° 2' 16 cycles

Run 10  $\mu$ l on 6% TBE

Digest Bste II 2 hr. 55° 90  $\mu$ l PCR product  
Eco RI 0.5 hr. 37° 10  $\mu$ l 10x RB 150 mM  
2  $\mu$ l Bste II  
2  $\mu$ l Eco RI

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Witnessed &amp; Understood by me,

Date

Invented by

Date

Recorded by

TITLE \_\_\_\_\_

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Tuesday 3/25/97

Miniscreen via PCR DD.2 5' ligations lig. #1

10 ml dH <sub>2</sub> O	x15
5 ml PCR Buffer	75
12 ml 1.25 mM dNTP's	180
0.5 M13f	7.5
0.5 M13r	7.5
22 ml dH <sub>2</sub> O	330
0.5 Tag	7.5

94° 1' 50° 1' 72° 1' 30 cycles

work up #4,5

Spleen new L.p.2  
wash

1x 6xSSC R.T  
2x 0.2xSSC  
1% SDS 42°  
2x 0.1xSSC  
1% SDS 42°

use microwave

expose O.N.

Quick ligation & transformation / IgG-DD.2

vectors

A A+B purified  
B C+D purified  
E prk5 Hu ITT, R Δ(R1-BsteII)

1 μl vector  
7 μl insert  
10 μl vial 1  
2 μl vial 2  
Mix  
1 μl vial 3

Inserts

1 DD.2 (sm.1-sm.4) Δ(R1-BsteII)  
2 DD.2 (sm.2-sm.4) Δ(R1-BsteII)

5' RT

Transform prk5 → 3L  
4,5

Transformation  
- 100 μl SM109  
20' 0° 45" 42° 2° 0°  
To Page No. \_\_\_\_\_

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Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

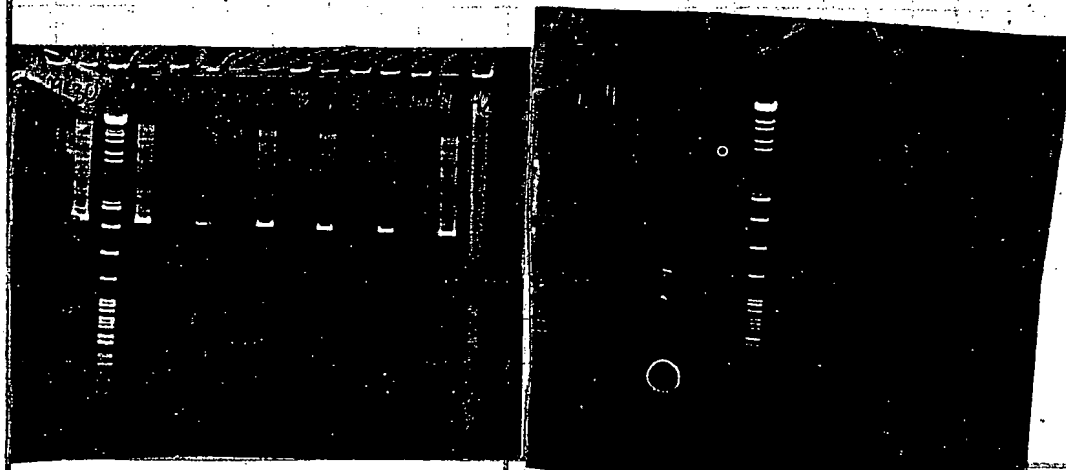
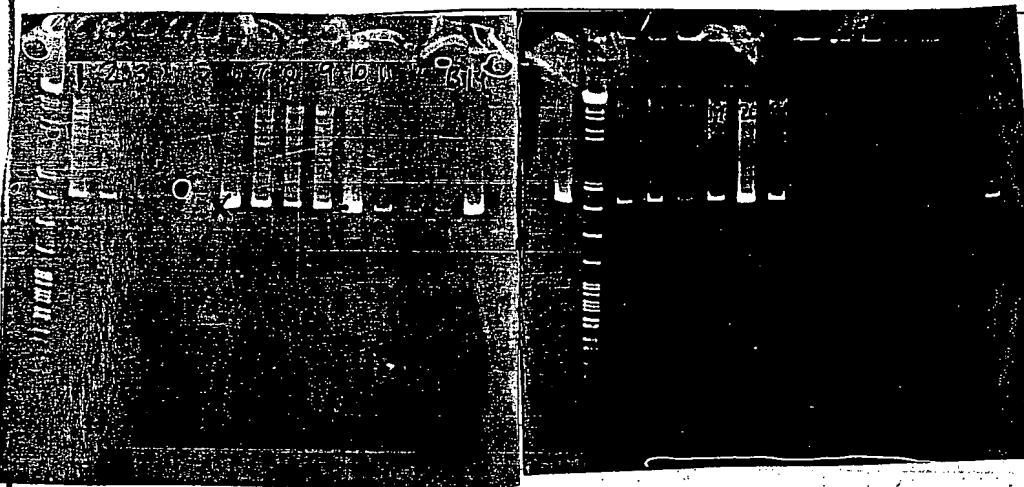
From Page No. \_\_\_\_\_

Wednesday 3/26/97PCR ligations 1g6-00.2

W8

No.	Signature		X 45 Rxns
1-8	1A	10 ul dH <sub>2</sub> O	540
9-21	2A	12 ul 1.25 mM dNTPs	22.5
22-26	B1	0.5 ul Sm2	22.5
27-30	B2	0.5 ul Sm4	22.5
31-35	E1	0.5 ul Tag	225
36-44	E2	5 ul PCR Buffer	990
		22 ul dH <sub>2</sub> O	

949'60" 1' 72" 3' 25 cycles



Submit 1g6-00.2  
#10, 31, 41 for  
sequencing

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

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Pick # 10, 20, 31, 41 for maxi prep

Maxi prep 4, 5, 3x prk5.

Thursday 3/27/97



Skubatch, Maya

#

REQUESTOR	cost center	phone	lab#	project #	required date
Skubatch, Maya	424	1849	10230	1110	3/31/97

FRAGMENTS

Fragment name	O.D.	Sequence	Speci
pcr.nik.1 24 mer	1	GCT CTG TGT TTG GTC TCT CTC AGG	
pcr.nik.3 24 mer	1	CCA CAC CTG GAG CCC AGC TGC CTG	
pcr.nik.3 24 mer	1	CCA CAC CTG GAG CCC AGC TGC CTG	
pcr.nik.2 24 mer	1	CGT GCT GCC CAG GTC TTG GCC AGG	
pcr.nik.4 24 mer	1	CGA GCA GGA AGG CTG GCT TTC CGG	

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Date \_\_\_\_\_

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Work up

Monday 3/31/97

Maxi prep

4x DD.2

new L1.1

new ligand 2.1

→ Submit for sequencing

PCR

DD.2-5' = try to pull 5' again using more external primers  
 see previous 2<sup>nd</sup> round PCR in ~~27233~~ 97-93. Use  
 Hu Lung Library

NIK = pull out of Hu Thymus library

PCR RxnsPrimers

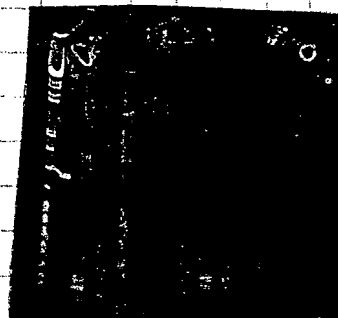
1	Hu Lung	Sm.1 - race. 1
2	"	Sm.1 - race. 2
3	"	Sm.1 - race. 3
4	"	Sm.2 - race. 1
5	"	Sm.2 - race. 2
6	"	Sm.2 - race. 3
7-12	(Hu fetal lung same)	
9	Hu Thymus	nik.1 nik.2

1 ul DNA  
 1 ul P1  
 1 ul P2  
 10 ul PCR  
 24 ul dNTPS  
 1 ul Tag  
 62 ul dH<sub>2</sub>O

1 cycle 94° 3'  
 35 cycles 94° 1' 60° 1' 72° 2'

Run on 6% gel

Pinky 182



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Witnessed &amp; Understood by me, \_\_\_\_\_

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Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_



TITLE \_\_\_\_\_

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Tuesday 4/1/97

5'-DD.2

Purify 1,2 on a column.

Ligation

SL

1A Hulang Sm.1 - Race.1

2A Hulang Sm.1 - Race.2

1 ul vector  
7 ul ~~vector~~ insert  
2 ul vial 2  
10 ul vial 1 mix  
1 ul vial 3  
5' R.T.

Long transformation

Screen MR3for / rev

DD.2-1g6

Digest DD.2 1g6 #41  
DD.2 1g6 #10  
DD.2 1g6 #31

1 ul DNA  
2 ul 150mM 10xRB  
6.5 ul BstE#  
~~0.5 ul B~~  
15 ul dH<sub>2</sub>O 55° 30'  
1 ul RI 37° 1 hr

Digest w/ RI BstEII



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Date

Invented by

Date

Recorded by

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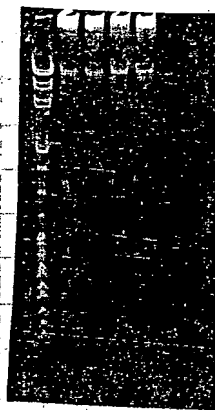
DD.2-IgG

Repeat ligation p.3

vector A, B, C

insert 1, 2

screen uIgG.4 - gm.1



NIK

PCR

Human Thymus

1 ul DNA

1 ul ~~PI~~ PI

1 ul P2

1 ul Taq

24 ul dNTPs

10 ul PCR Buffer

62 ul dH<sub>2</sub>O

Primers

#1 NIK 1 + NIK 2

#2 NIK 3 + NIK 4

#3 NIK 1 + NIK 4

94° 1' 58° 1' 72° 3' 35 cycles

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Witnessed &amp; Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

TITLE \_\_\_\_\_

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Wednesday 4.2.97

PCR Screening

#1-48 (196- DD.2 samples)

1-5	1A
6-14	2A
15-25	1B
26-28	2B
29-38	1E
39-44	2E

10 ml dH<sub>2</sub>O + plug  
0.5 IgG.4  
0.5 sm.1  
0.5 Tag  
5 PCR Buffer  
12 dNTP's  
22 dH<sub>2</sub>O

~~x 48~~  
~~24~~  
~~24~~  
~~24~~  
~~240~~  
~~876~~  
~~1056~~

50	25
25	12.5
25	12.5
25	12.5
250	125
800	300
1100	550

#49-72 (DD.2 - 5') *Hy Lung*

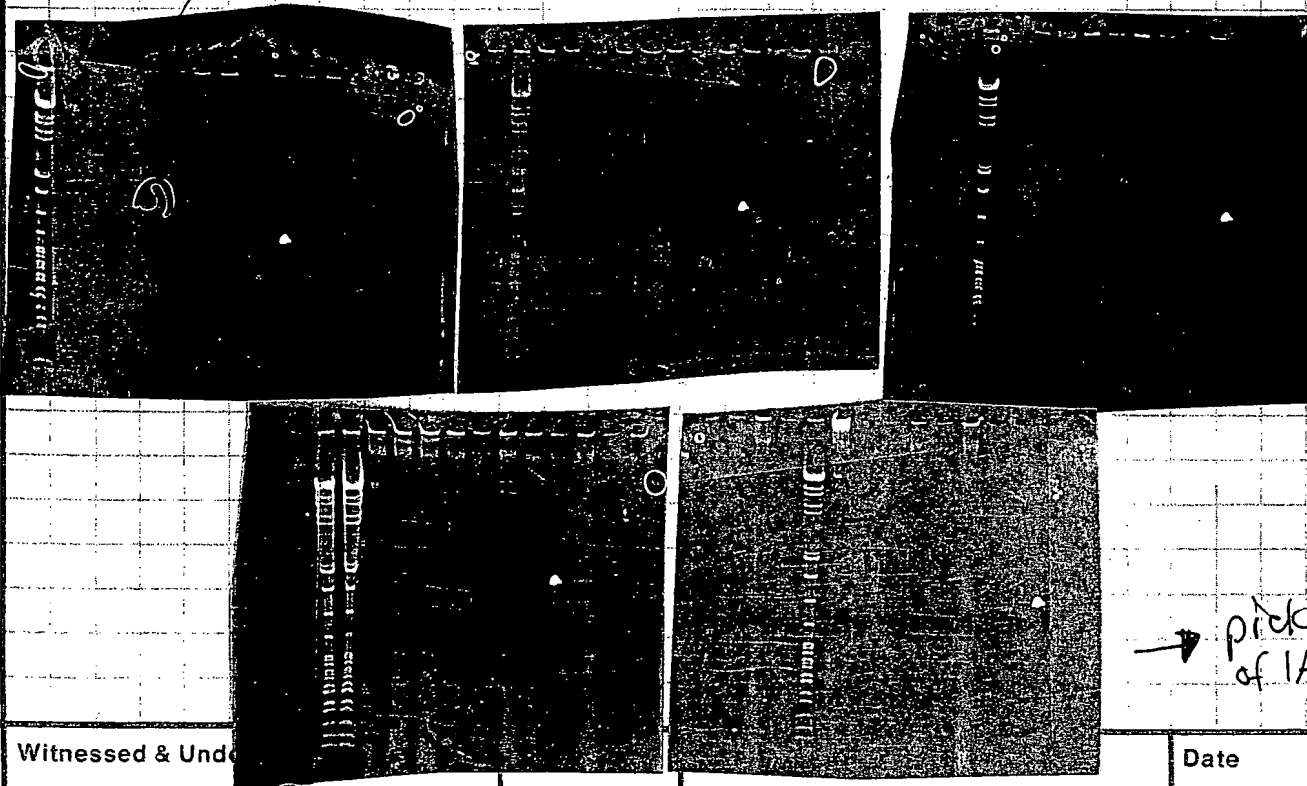
#49-60	1A
61-72	2A

(M13 for M13 rev)

same ↑

94°1' 60°1' 72°3' 35 cycles

→ plasmid prep of #5 "A+B / sm.1 - sm.4"  
digest w/ P1 - BstEII



→ pick 96 of 1A for miniscreen  
To Page No. \_\_\_\_\_

Witnessed & Under \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

TITLE Thursday 4/3/97Book No. 27236

From Page No. \_\_\_\_\_

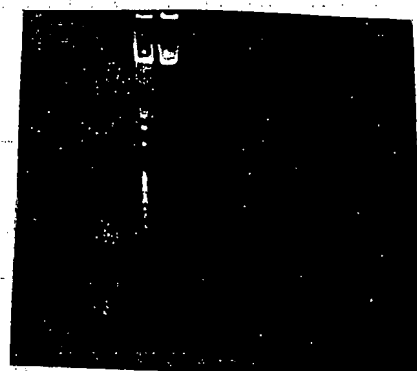
Digest  
SVI-12.1 #A7

- 1) RI
- 2) RI-H3
- 3) Bam H1 - Hind III
- 4) Xba - Hind III

Maxi Prep IgG-00.2 #5

Digest (RI-BstEII)

3 ul DNA  
2 ul 10x RB  
1 ul RI  
1 ul BstEII  
13 ul D<sub>2</sub>O

IgG-00.2  
Long ligation

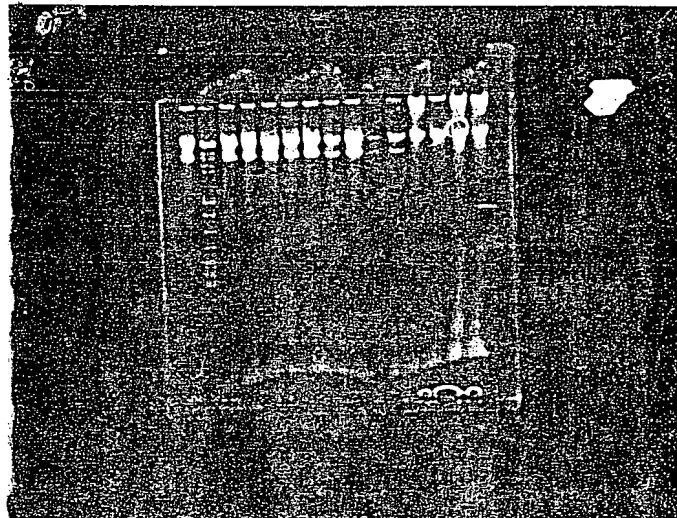
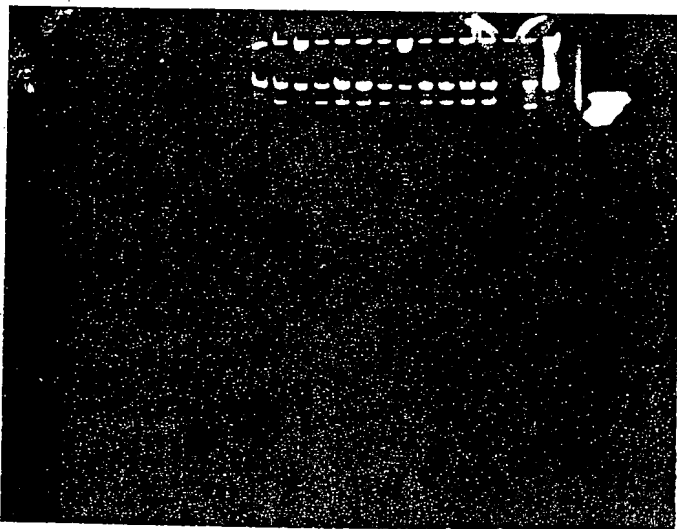
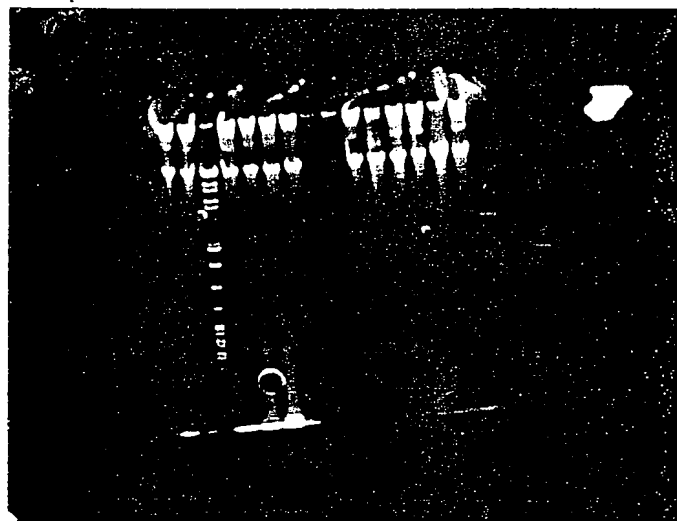
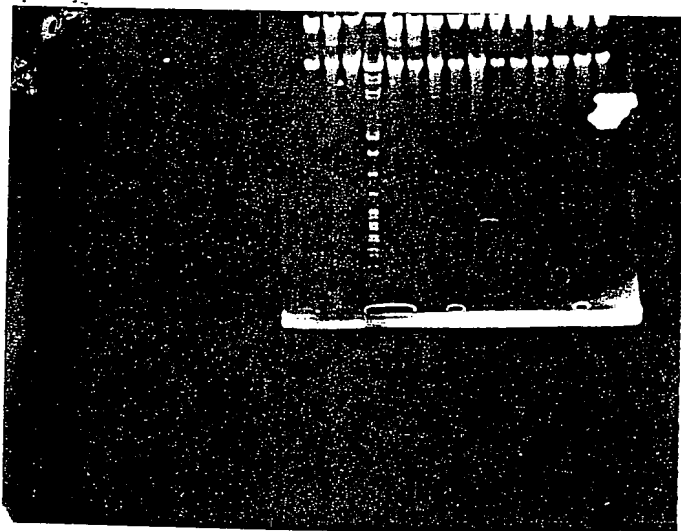
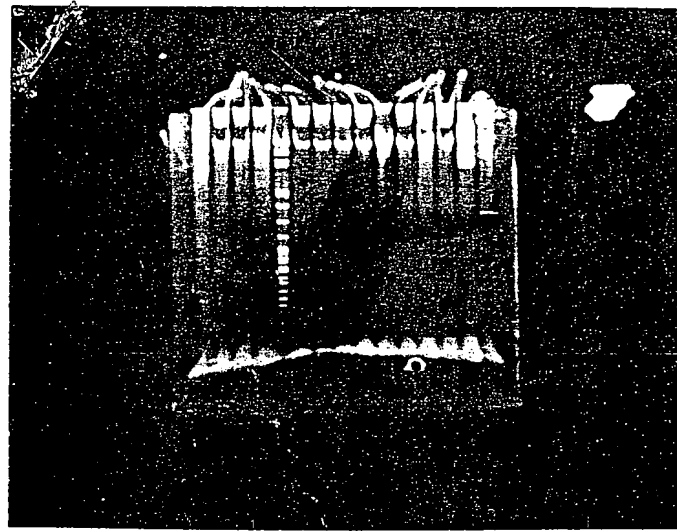
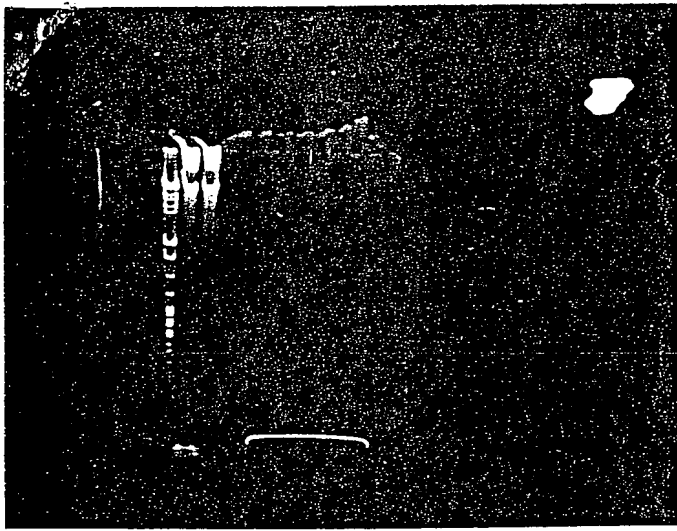
1 ul Ligase  
1 ul 10x LB  
7 ul ~~vector~~ insert  
1 ul vector

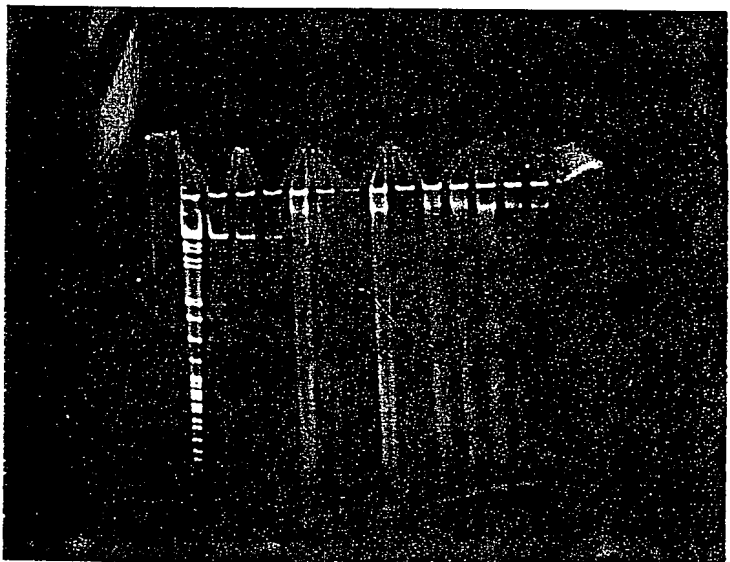
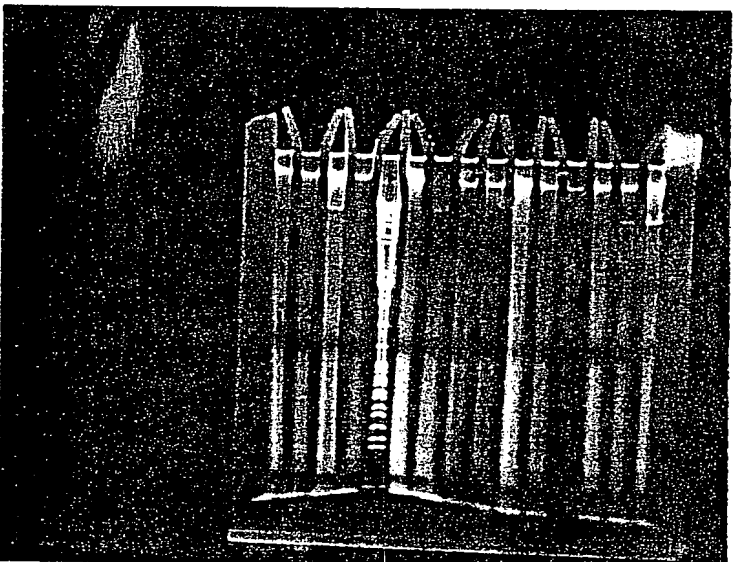
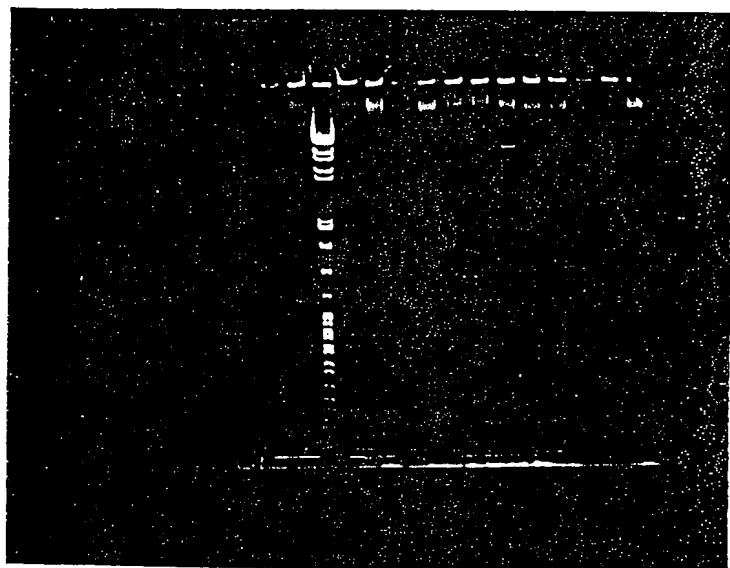
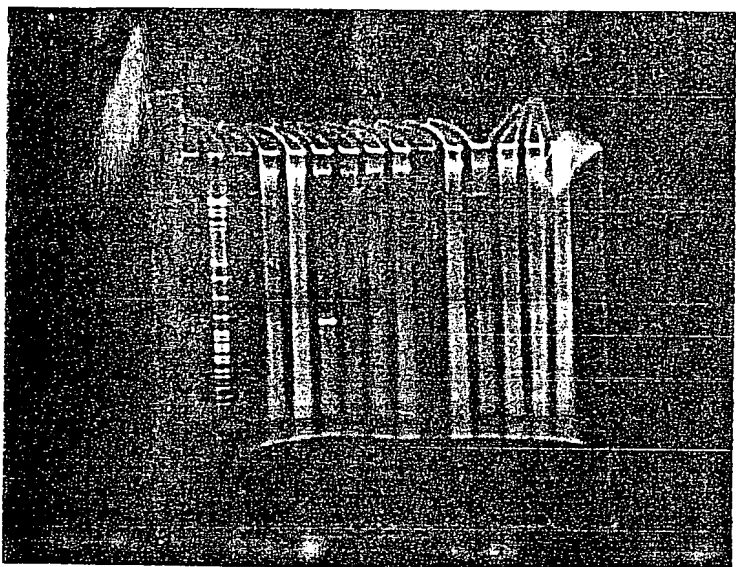
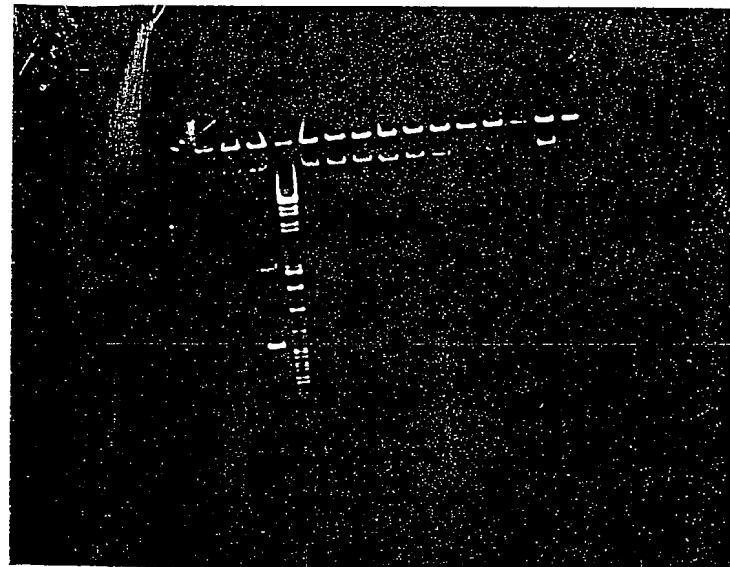
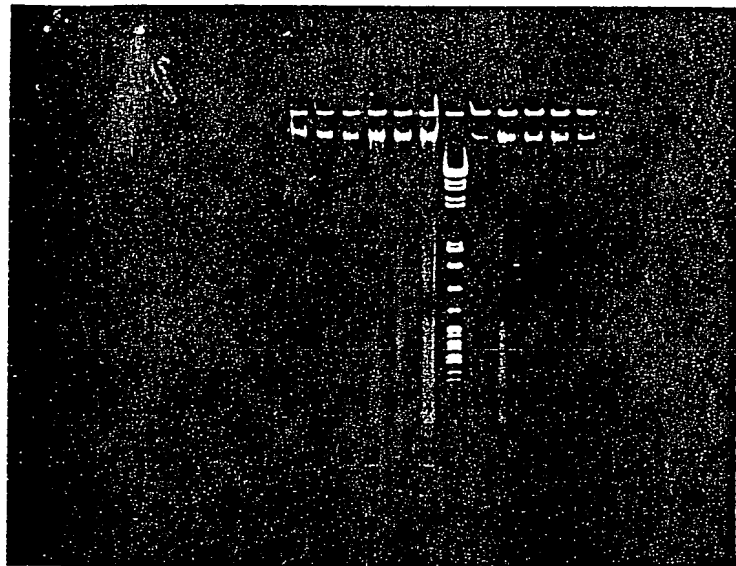
Miniscreen 5'00.2 (lig 1A)  
x100

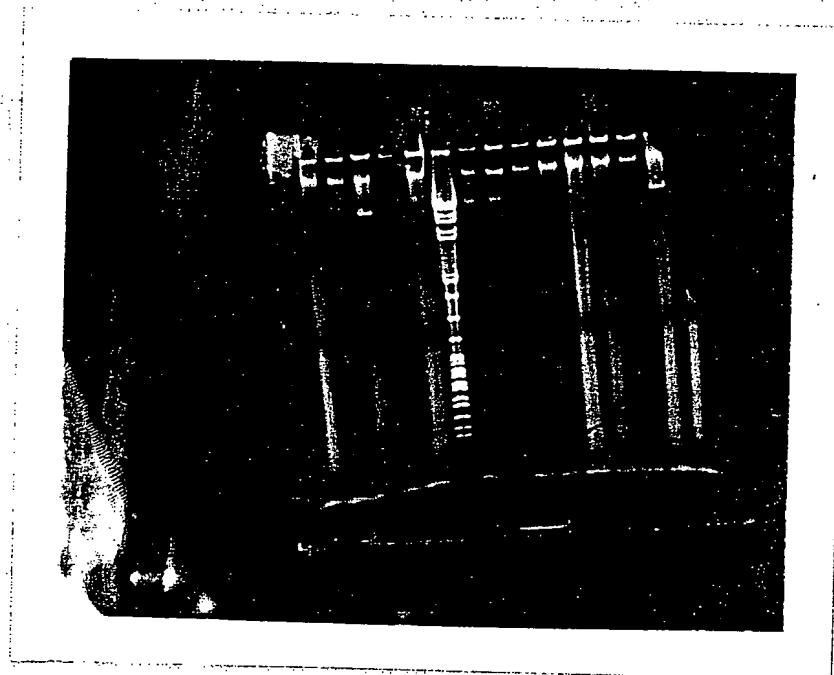
10 ul dH <sub>2</sub> O	
12 ul dNTP	1200
5 10x PCR Buffer	500
0.5 M13f	50
0.5 M13r	50
0.5 Taq	50
22 dA <sub>20</sub>	2200

Witnessed &amp; Understood by me,

Date







From Page No. \_\_\_\_\_

DD.2-IgG ConstructPCR the DD.2 Insert

(1:1000) 1.0 ~~ul~~ 002.1 DNA  
 1.0 ul sm.1 / sm.2  
 1.0 ul sm.4  
 10 ul 10x PCR Buffer  
 24 ul 1.25mM dNTP  
 63 ul dH<sub>2</sub>O  
 1 ul Tag

94° 3' 1 cycle  
 94° 1' 60° 1' 72° 2' 16 cycles

PCR Product Digestion w/ BstEII and EcoRI

90 ul PCR Product  
 10 ul 10x 150mM RBS  
 2 ul BstEII

55° 2 hrs

2 ul EcoRI

37° 0.5N

QUICK TRANSFORMATION

~~HRP~~ → Hu IFN $\gamma$ R - beta subunit gene + Hu IgG<sub>1</sub> #4348  
 Mu IFN $\gamma$ R beta subunit gene + h IgG<sub>1</sub>

1 ul DNA, 100 ul JM109  
 20' 0° 45" 42° 2' 0°

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Date

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Date

Recorded by



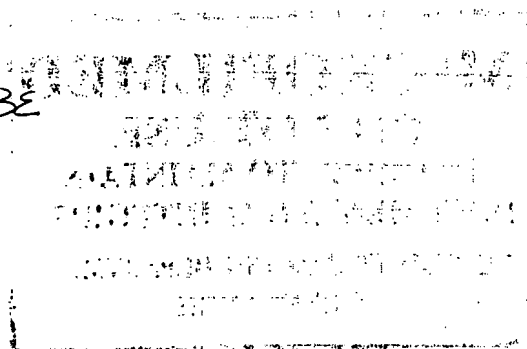
From Page No. \_\_\_\_\_

DD.2-5'End

PCR Hujung

<u>Rxn#</u>	<u>Primers</u>			
1	prk.rev	-	DD.Pac.1	1 ul cDNA
2	SM.1	-	DD.Pac.1	1 ul P1
3	SM.2	-	DD.Pac.1	1 ul P2
4	prk.rev	-	DD.Pac.2	10 ul 10x PCR Buffer
5	SM.1	-	DD.Pac.2	24 ul d.25 dNTPS
6	SM.2	-	DD.Pac.2	62 ul dH <sub>2</sub> O
7	prk.rev	-	DD.Pac.3	1 ul Tag
8	SM.1	-	DD.Pac.3	
9	SM.2	-	DD.Pac.3	
94°3' 1 cycle				
94°1' 60°1' 72°2' 35 cycles				

Run 10ul on 6% TBE  
Refrigerate



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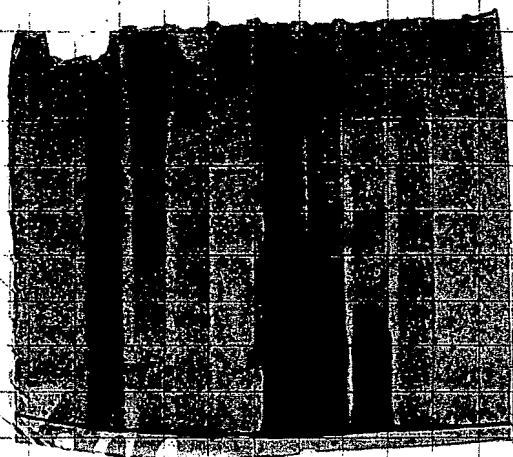
Invented by

Date

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1 3 5 std 2 4 6

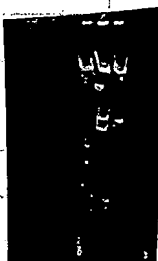


Mutagenesis  
pick 4x scots

DD-2 ECD-Flag  
Quick Transformation 1000ml → 4L

Newligands / Digest and Sequencing

16 ml dH<sub>2</sub>O  
2 ml 10x 150mM RB  
1 ml H<sub>3</sub>  
1 ml R1



37° 45'  
Run on GXTBE

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Invented by \_\_\_\_\_

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Thursday 4.24.97

Maxiprep

M1 3.4

M1 6.31

M2 29.43

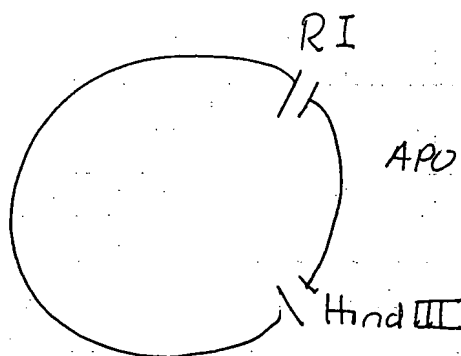
M2 27.2

DD.2 - ECD FLAG

DD.2 - ECD FLAG

Blue Script - DD.2 ConstructDigest3  $\mu$ l DD.20.5  $\mu$ l RI0.5  $\mu$ l H32  $\mu$ l 150 mM RB14  $\mu$ l dH<sub>2</sub>O2  $\mu$ l Blue Script II KS+0.5  $\mu$ l RI0.5  $\mu$ l H32  $\mu$ l 150 mM RB14  $\mu$ l dH<sub>2</sub>O

37° O.N



To Page No. \_\_\_\_\_

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Invented by \_\_\_\_\_

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TNFR2p2 (30942)  
 Quick transform → 500ml

Friday 4.25.97

redigest 100 ul Rn

3 ul DD.2  
 1 ul R1  
 1 ul H3  
 10 ul 10x 150mM RB  
 85 ul dH<sub>2</sub>O

2 ul BScript-ksr  
 1 ul R1  
 1 ul H3  
 10 ul 10x 150mM RB  
 86 ul dH<sub>2</sub>O

2hrs 370

maxi prep TNFRp2



Skubatch, Maya

#

April 24, 1997

REQUESTOR	cost center	phone	lab#	project #	required date	
Skubatch, Maya	424	1849	10230	1110	4/28/97	7006

#### FRAGMENTS

Fragment name	O.D.	Sequence	Special Instructions
TNFR2p.IgG 36 mer	1	GCA TGT GTG AGT TTT GTC GTG CAC AGG GAG GAA GCG	DNA reference:
TNFR2p.IgG.probe 16 mer	1	GTT TTG TCG TGC ACA G	DNA reference:
HVEM.IgG 36 mer	1	GCA TGT GTG AGT TTT GTC CCC AGC TCC GGC CTT CGT	DNA reference:
HVEM.IgG.probe 16 mer	1	GTT TTG TCC CCA GCT C	DNA reference:

|||

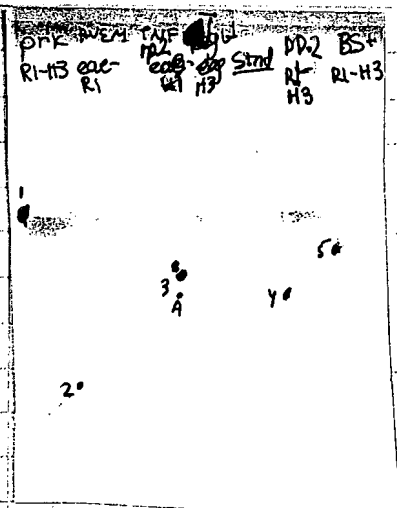
Project No. \_\_\_\_\_  
Book No. 27236

23

TITLE \_\_\_\_\_

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Run Digest from Friday



Cut and refrigerate.

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Witnessed & Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

HVE M-IgG / APO2-BlueScreen

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## Quick Ligation Kit

SLA 1ul prk 5	1A	1ul prk 5	R1-H3	<del>2A</del> Same
7ul dH <sub>2</sub> O		3.5ul IgG 3A	rag-H3	IgG 3B
1ul ligase buffer vial 1		3.5ul HVE M eae-R1		same
1ul ligase		1ul ligase buffer vial 1		↓
2ul vial 2		1ul ligase		
		2ul vial 2		

~~2A 1ul prk~~

SLB 1ul BS +	1B	1ul BS
7ul dH <sub>2</sub> O		7ul DO.2
1ul LB vial 1		1ul LB vial 1
1ul ligase		1ul ligase
2ul vial 2		2ul vial 2

RT 10'

Transform 100ul JM109. 20° 45" 42°, 2' 0"

45° 37' on wheel

plate

Run fragments on gel

## Quick Transformation

VARNA 500ml  
prk 7 500ml  
APO2 10g #72 1L  
DR 4 10g 1L

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by \_\_\_\_\_

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## Long ligations

SLA pet 19b (Xho - Nco)

1A pet 19b - mu APO2L

SLB pet 19b (Nde - BamHI)

1B pet 19b - APO2L mut #10

2B pet 19b - APO2L mut #76

SLC prkS (RI - H3)

1C prkS - IgG'3A' - HVEM (RI - cae - H3)

2C prkS - IgG'3B' - HVEM (RI - cae - H3)

3C prkS - HVEM-Flag (RI - H3) 4/29

4C prkS - TNFR2-rp-Flag (RI - H3) 4/29

SLD BlueScript (RI - H3)

1D BS - APO2L/PD2 (RI - H3)

all

Exn's all

7ul vector

7ul insert/dH<sub>2</sub>O

1ul 10XLB

1ul Ligase

O.N. 14°

Rapid Ligations of muAPO2

1ul vial 1

2ul vial 2

1ul vector

1ul insert

1ul ligase vial 3

## TNFR2 rp plasmid

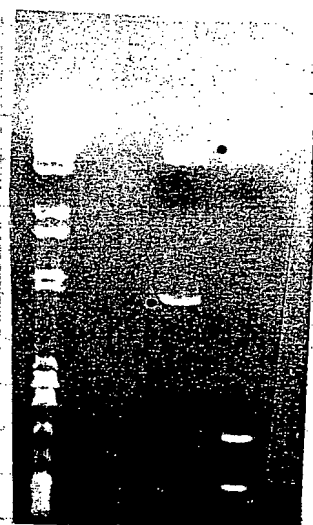
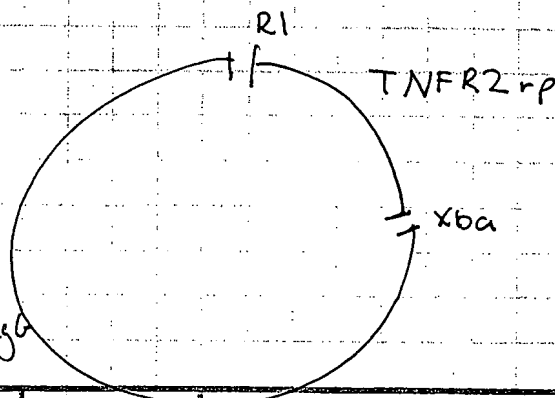
## Digest

Bul prkS HuINxR - HuIgG<sub>1</sub> / or TNFR2rp (4/25/97)

10ul 10x 100mM RB

2ul Xba

2ul EcoRI

83ul dH<sub>2</sub>OprkS  
HuINxR-HuIgG<sub>1</sub>

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Witnessed &amp; Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

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Friday 5.2.97

## PCR ligations

		Rxn.	x 75
#1-24	pet 19b mu APO 2L (ligation 1A p.29)	10 ul dH <sub>2</sub> O + colony	750
#25-48	pet 19b APO 2L mut c→s #10 (lig 1B)	5 ul 10xPCR	375
#49-72	pet 19b APO 2L mut c→s #76 (lig 2B)	12 ul dNTPS	900
		0.5 ul prk for	37.5
		0.5 ul prk rev	37.5
		22 ul dH <sub>2</sub> O	1650
		0.5 ul Tag	37.5
73	prk 1gG HVEM (lig 1C) <sup>primers</sup> (HVEM - 1gG4)		
74	DD.2 (prk for - prk rev)		
94°1' 50°1' 72°3' 30 cycles			

NOTHING CAME OUT IN THE GELS

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Invented by

Date

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AP02/scrip

Digest	DD.2/AP02	2ul
	PvuI	1ul
	EcoRI	1ul
	HindIII	1ul
	100mM	10ul
	dH <sub>2</sub> O	85ul

2h 37°

add 50ul  $\phi$ -OH, vortex  
 50ul CHCl<sub>3</sub>, vortex  
 14K 5',  
 take off 100ul from top layer  
 add 10ul 3M Na Acetate  
 200ul 100% EtOH  
 Place on ice until all liquid dries up.  
 14K 5',  $\nearrow$   
 100ul 70% EtOH  
 14K 5'  $\nearrow$   
 30' speed vac  
 120ul dH<sub>2</sub>O.

(for ligation into SGA plasmid)

Purification

TNFA2rp / PRK5 (Xba-R1)

Qiagen minicolumn.

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Date

Invented by

Date

Recorded by

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Monday 5.5.97Rat Kidney library titer  $\sim 1 \times 10^9$   
plate 20 x 50 x 10<sup>-5</sup> using 0.5 ml PSB, 50  $\mu$ l cDNA, 0.5 ml C600, 9 ml ~~ADH~~Miniprep

- #1 prK5-lgG-HVEM
- #2-24 prK5-TNFR2rp (Xba-R1)
- #25-48 pet19b-muAPO2L
- #49-60 pet19b APO2L (L $\rightarrow$ S) #10
- #61-72 pet19b APO2L (L $\rightarrow$ S) #76

miniscreen  
Digests

R1-H3  
R1-Xba  
Nco-Xho  
Nde-Bam  
Nde-Bam

Ligation / Quick Ligation of Blue Script Vector + APO2

1  $\mu$ l Blue Script  
7  $\mu$ l APO2 / dH<sub>2</sub>O  
10  $\mu$ l vial 1 (QL)  
2  $\mu$ l vial 2 (QL)  
1  $\mu$ l vial 3 (QL)

1  $\mu$ l Blue Script  
7  $\mu$ l APO2 / dH<sub>2</sub>O  
1  $\mu$ l 10xLB (L.L)  
1  $\mu$ l 10mM ATP (L.L)  
1  $\mu$ l vial 3

O.N. 14°

5'R.T.

Transform, use 100  $\mu$ l JM109 20'0°, 45'42°, 2'0°

Plate on LB + Carls

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

Page #1

Subject: rhserver@shgc: completed two point calculations

messageID: 199705051846.LAA21872.

```
prh_score: 00000 00000 00000 00000 00010 00000 00010 00001 10100 11000 10001 00001 00010 10100 00000 01000
000
```

NOTEBOOK NO. 26466  
ISSUED TO Lakshmi Ramakrishnan  
ON [REDACTED] 19 [REDACTED]  
DEPARTMENT Mol. Biology  
RETURNED 19

—SCIENTIFIC NOTEBOOK CO.—  
2831 LAWRENCE AVE.  
P.O. BOX 238  
STEVENSVILLE, MI 49127  
616-429-8285

ADE- 29  
USSN 10/052,798

From Page No. \_\_\_\_\_

## PCR Analysis of cDNA's from Incyte Database

picked up by Austin Gueney

Novel IGFBP/GDF3 homolog - IN 206112

Novel FGF8 homolog - IN 313182

" " " - IN 843193

	1x	3x
1x buffer	3	114
dNTPs	8	114
dH <sub>2</sub> O	22.1	839.8
Primers	3/3	3.6/3.6
Enzyme	3	11.4

\*DNA (100ng) 11

\*Diluted all ssDNA's to have  
stock of 1x/1

1. F. Kidney < 2.8kb
2. F. Kidney > 2.8kb
3. Liver
4. F. Lung < 2.8kb
5. F. Lung > 2.8
6. Placenta < 2.8
7. Placenta > 2.8
8. Retina
9. Salivary gland
10. Small Intestine
11. Thyroid

made up a master mix of klenow buffer, dNTPs & dH<sub>2</sub>O to  
do similar PCR analysis for other Incyte clones.

	50x
10x buffer	150
dNTP	150
dH <sub>2</sub> O	1130

+ 100ng/30x of every ssDNA library.

Results from above screen:

Screen F. lung &lt; 2.8 kb library for all three clones above.

X X X X X X X X

Scott - brought a Pancreas library - to make  
ssDNA - to screen for a novel protein - homolog  
of Apo 3 - has Death Domain- started to make ssDNA - Pancreas on [redacted] & completed on  
[redacted]. Plated 10<sup>6</sup> cells.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

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Washes

Filters from \_\_\_\_\_, wash as usual

2x @ RT, 2x SSY, 1x SDS 15' each

2x @ 42°C " " "

Expose & develop ON for DD from \_\_\_\_\_  
5 hr for rescues from \_\_\_\_\_

Result: - New Rescues

DD - positives about 3 or 4  
background high, difficult to  
distinguish true pos from  
false ones.

18882 - No positives!

Plate for 2° Screen from DD - 4 positives  
- ~~plated~~ picked colony into 100x LB/carb.  
2° DD K.1 thru K.4  
- plated 5.1 of  $10^{-2}$  of eachOld Rescue of DDA positives - cannot pick single's  
- so plate 2° Screen do same as above  
pick into 100x & plate ~ 3-5.1 of  $10^{-2}$ .  
2° DD P.1 → P.42° Screen plates DD K.1 - K.4 were too dense for 2°  
plate again! 1-2.1 of  $10^{-3}$  dilutionScreen DD P.1 - P.4 - lift, process, prehyb & hyb for ~  
3-4 hrs- Wash as usual - filters quite hot! ~~wait to expose~~  
get short exposure for ~ 1 hr - picked 1 positive for each

Witnessed &amp; Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by \_\_\_\_\_

for miniprep's

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

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DD - 82° Screen from Pancreas

- Picked 4 positives, 1 from each 2° screened & put up minipreps & also for large scale cultures for Scott/Avi's lab to put into assay.
- Miniprep Analysis by PCR & Restriction Digest.

PCR - as usual with all 4 primer sets

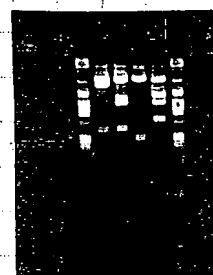
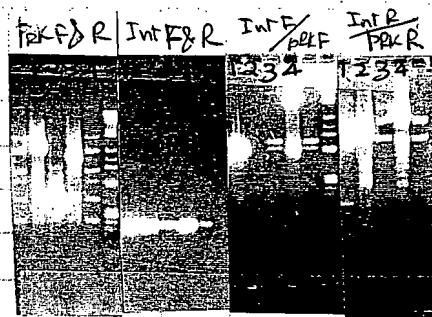
PRK F&R  
Int F&RInt F&PRKF  
Int R&PRKR

Conditions:

95° }  
59° } 3  
72° }

95° }  
57° } 3  
72° }

95° }  
55° } 20 cycles  
72° }



XbaI digest

All clones PCR w/ Internal Primers to give expected product size about 1500bp

	PRK F&R	Int F&R	Int F/PRKF	Int R/PRKR	XbaI digest
DDP.1	multiple bands?	~1500bp	~700bp		~2kb
P.2	~500bp	"	none		~2kb
P.3	~1.2kb	"	~700		~500
P.4	multiple bands	"	~1.9kb		>3kb?

given for sequencing by Scott on [redacted]

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Recorded by



#

## FRAGMENTS

**O.D.**

### Special Instructions

**ADE- 30**  
USSN 10/052,798





#

1

6258

**ADE- 31**  
USSN 10/052,798



#

REQUESTOR	cost center	phone	lab#	project #	required date	
Marsters, Scot	424	5747	10230	1110	3/21/97	6496

[illegible]

**ADE- 32**  
USSN 10/052,798

Apo-2 Receptor

FIELD OF THE INVENTION

The present invention relates generally to the identification, isolation, and recombinant production of novel polypeptides, designated herein as "Apo-2".

BACKGROUND OF THE INVENTION

Apoptosis or "Programmed Cell Death"

Control of cell numbers in mammals is believed to be determined, in part, by a balance between cell proliferation and cell death. One form of cell death, sometimes referred to as necrotic cell death, is typically characterized as a pathologic form of cell death resulting from some trauma or cellular injury. In contrast, there is another, "physiologic" form of cell death which usually proceeds in an orderly or controlled manner. This orderly or controlled form of cell death is often referred to as "apoptosis" [see, e.g., Barr et al., Bio/Technology, 12:487-493 (1994); Steller et al., Science, 267:1445-1449 (1995)]. Apoptotic cell death naturally occurs in many physiological processes, including embryonic development and clonal selection in the immune system [Itoh et al., Cell, 66:233-243 (1991)]. Decreased levels of apoptotic cell death have been associated with a variety of pathological conditions, including cancer, lupus, and herpes virus infection [Thompson, Science, 267:1456-1462 (1995)]. Increased levels of apoptotic cell death may be associated with a variety of other pathological conditions, including AIDS, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, multiple sclerosis, retinitis pigmentosa, cerebellar degeneration, aplastic anemia, myocardial infarction, stroke, reperfusion injury, and toxin-induced liver disease [see, Thompson, supra].

Apoptotic cell death is typically accompanied by one or more characteristic morphological and biochemical changes in cells, such as

5 condensation of cytoplasm, loss of plasma membrane microvilli,  
segmentation of the nucleus, degradation of chromosomal DNA or loss of  
mitochondrial function. A variety of extrinsic and intrinsic signals  
are believed to trigger or induce such morphological and biochemical  
cellular changes [Raff, Nature, 356:397-400 (1992); Steller, supra;  
10 Sachs et al., Blood, 82:15 (1993)]. For instance, they can be triggered  
by hormonal stimuli, such as glucocorticoid hormones for immature  
thymocytes, as well as withdrawal of certain growth factors [Watanabe-  
Fukunaga et al., Nature, 356:314-317 (1992)]. Also, some identified  
oncogenes such as *myc*, *rel*, and *E1A*, and tumor suppressors, like *p53*,  
15 have been reported to have a role in inducing apoptosis. Certain  
chemotherapy drugs and some forms of radiation have likewise been  
observed to have apoptosis-inducing activity [Thompson, supra].

#### TNF Family of Cytokines

Various molecules, such as tumor necrosis factor- $\alpha$  ("TNF- $\alpha$ "),  
20 tumor necrosis factor- $\beta$  ("TNF- $\beta$ " or "lymphotoxin"), CD30 ligand, CD27  
ligand, CD40 ligand, OX-40 ligand, 4-1BB ligand, Apo-1 ligand (also  
referred to as Fas ligand or CD95 ligand), and Apo-2 ligand (also  
referred to as TRAIL) have been identified as members of the tumor  
necrosis factor ("TNF") family of cytokines [See, e.g., Gruss and Dower,  
25 Blood, 85:3378-3404 (1995); Wiley et al., Immunity, 3:673-682 (1995);  
Pitti et al., J. Biol. Chem., 271:12687-12690 (1996); WO 97/01633  
published January 16, 1997]. Among these molecules, TNF- $\alpha$ , TNF- $\beta$ , CD30  
ligand, 4-1BB ligand, Apo-1 ligand, and Apo-2 ligand (TRAIL) have been  
reported to be involved in apoptotic cell death. Both TNF- $\alpha$  and TNF- $\beta$   
30 have been reported to induce apoptotic death in susceptible tumor cells  
[Schmid et al., Proc. Natl. Acad. Sci., 83:1881 (1986); Dealtry et al.,  
Eur. J. Immunol., 17:689 (1987)]. Zheng et al. have reported that TNF- $\alpha$   
is involved in post-stimulation apoptosis of CD8-positive T cells [Zheng  
et al., Nature, 377:348-351 (1995)]. Other investigators have reported  
35 that CD30 ligand may be involved in deletion of self-reactive T cells in  
the thymus [Amakawa et al., Cold Spring Harbor Laboratory Symposium on  
Programmed Cell Death, Abstr. No. 10, (1995)].

Mutations in the mouse Fas/Apo-1 receptor or ligand genes  
(called *lpr* and *gld*, respectively) have been associated with some  
40 autoimmune disorders, indicating that Apo-1 ligand may play a role in

5 regulating the clonal deletion of self-reactive lymphocytes in the  
periphery [Krammer et al., Curr. Op. Immunol., 6:279-289 (1994); Nagata  
et al., Science, 267:1449-1456 (1995)]. Apo-1 ligand is also reported  
to induce post-stimulation apoptosis in CD4-positive T lymphocytes and  
in B lymphocytes, and may be involved in the elimination of activated  
10 lymphocytes when their function is no longer needed [Krammer et al.,  
supra; Nagata et al., supra]. Agonist mouse monoclonal antibodies  
specifically binding to the Apo-1 receptor have been reported to exhibit  
cell killing activity that is comparable to or similar to that of TNF- $\alpha$   
[Yonehara et al., J. Exp. Med., 169:1747-1756 (1989)].

#### 15 TNF Family of Receptors

Induction of various cellular responses mediated by such TNF  
family cytokines is believed to be initiated by their binding to  
specific cell receptors. Two distinct TNF receptors of approximately  
55-kDa (TNFR1) and 75-kDa (TNFR2) have been identified [Hohman et al.,  
20 J. Biol. Chem., 264:14927-14934 (1989); Brockhaus et al., Proc. Natl.  
Acad. Sci., 87:3127-3131 (1990); EP 417,563, published March 20, 1991]  
and human and mouse cDNAs corresponding to both receptor types have been  
isolated and characterized [Loetscher et al., Cell, 61:351 (1990);  
Schall et al., Cell, 61:361 (1990); Smith et al., Science, 248:1019-1023  
25 (1990); Lewis et al., Proc. Natl. Acad. Sci., 88:2830-2834 (1991);  
Goodwin et al., Mol. Cell. Biol., 11:3020-3026 (1991)]. Extensive  
polymorphisms have been associated with both TNF receptor genes [see,  
e.g., Takao et al., Immunogenetics, 37:199-203 (1993)]. Both TNFRs  
share the typical structure of cell surface receptors including  
30 extracellular, transmembrane and intracellular regions. The  
extracellular portions of both receptors are found naturally also as  
soluble TNF-binding proteins [Nophar, Y. et al., EMBO J., 9:3269 (1990);  
and Kohno, T. et al., Proc. Natl. Acad. Sci. U.S.A., 87:8331 (1990)].  
The cloning of recombinant soluble TNF receptors was reported by Hale et  
35 al. [J. Cell. Biochem. Supplement 15F, 1991, p. 113 (P424)].

The extracellular portion of type 1 and type 2 TNFRs (TNFR1  
and TNFR2) contains a repetitive amino acid sequence pattern of four  
cysteine-rich domains (CRDs) designated 1 through 4, starting from the  
NH<sub>2</sub>-terminus. Each CRD is about 40 amino acids long and contains 4 to 6  
40 cysteine residues at positions which are well conserved [Schall et al.,  
supra; Loetscher et al., supra; Smith et al., supra; Nophar et al.,

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5 supra; Kohno et al., supra]. In TNFR1, the approximate boundaries of the four CRDs are as follows: CRD1- amino acids 14 to about 53; CRD2- amino acids from about 54 to about 97; CRD3- amino acids from about 98 to about 138; CRD4- amino acids from about 139 to about 167. In TNFR2, CRD1 includes amino acids 17 to about 54; CRD2- amino acids from about 55 to about 97; CRD3- amino acids from about 98 to about 140; and CRD4- amino acids from about 141 to about 179 [Banner et al., Cell, 73:431-435 (1993)]. The potential role of the CRDs in ligand binding is also described by Banner et al., supra.

15 A similar repetitive pattern of CRDs exists in several other cell-surface proteins, including the p75 nerve growth factor receptor (NGFR) [Johnson et al., Cell, 47:545 (1986); Radeke et al., Nature, 325:593 (1987)], the B cell antigen CD40 [Stamenkovic et al., EMBO J., 8:1403 (1989)], the T cell antigen OX40 [Mallet et al., EMBO J., 9:1063 (1990)] and the Fas antigen [Yonehara et al., supra and Itoh et al., supra]. CRDs are also found in the soluble TNFR (sTNFR)-like T2 proteins of the Shope and myxoma poxviruses [Upton et al., Virology, 160:20-29 (1987); Smith et al., Biochem. Biophys. Res. Commun., 176:335 (1991); Upton et al., Virology, 184:370 (1991)]. Optimal alignment of these sequences indicates that the positions of the cysteine residues are well conserved. These receptors are sometimes collectively referred to as members of the TNF/NGF receptor superfamily. Recent studies on p75NGFR showed that the deletion of CRD1 [Welcher, A.A. et al., Proc. Natl. Acad. Sci. USA, 88:159-163 (1991)] or a 5-amino acid insertion in this domain [Yan, H. and Chao, M.V., J. Biol. Chem., 266:12099-12104 (1991)] had little or no effect on NGF binding [Yan, H. and Chao, M.V., supra]. p75 NGFR contains a proline-rich stretch of about 60 amino acids, between its CRD4 and transmembrane region, which is not involved in NGF binding [Peetre, C. et al., Eur. J. Hematol., 41:414-419 (1988); Seckinger, P. et al., J. Biol. Chem., 264:11966-11973 (1989); Yan, H. and Chao, M.V., supra]. A similar proline-rich region is found in TNFR2 but not in TNFR1.

40 Itoh et al. disclose that the Apo-1 receptor can signal an apoptotic cell death similar to that signaled by the 55-kDa TNFR1 [Itoh et al., supra]. Expression of the Apo-1 antigen has also been reported to be down-regulated along with that of TNFR1 when cells are treated with either TNF- $\alpha$  or anti-Apo-1 mouse monoclonal antibody [Krammer et

5 al., supra; Nagata et al., supra]. Accordingly, some investigators have hypothesized that cell lines that co-express both Apo-1 and TNFR1 receptors may mediate cell killing through common signaling pathways [Id.]

10 The TNF family ligands identified to date, with the exception of lymphotoxin- $\alpha$ , are type II transmembrane proteins, whose C-terminus is extracellular. In contrast, the receptors in the TNF receptor (TNFR) family identified to date are type I transmembrane proteins. In both the TNF ligand and receptor families, however, homology identified between family members has been found mainly in the extracellular domain ("ECD"). Several of the TNF family cytokines, including TNF- $\alpha$ , Apo-1 ligand and CD40 ligand, are cleaved proteolytically at the cell surface; the resulting protein in each case typically forms a homotrimeric molecule that functions as a soluble cytokine. TNF receptor family proteins are also usually cleaved proteolytically to release soluble receptor ECDs that can function as inhibitors of the cognate cytokines.

20 Recently, other members of the TNFR family have been identified. In Marsters et al., Curr. Biol., 6:750 (1996), investigators describe a full length native sequence human polypeptide, called Apo-3, which exhibits similarity to the TNFR family in its extracellular cysteine-rich repeats and resembles TNFR1 and CD95 in that it contains a cytoplasmic death domain sequence [see also Marsters et al., Curr. Biol., 6:1669 (1996)]. Apo-3 has also been referred to by other investigators as DR3, wsl-1 and TRAMP [Chinnaiyan et al., Science, 274:990 (1996); Kitson et al., Nature, 384:372 (1996); Bodmer et al., Immunity, 6:79 (1997)].

30 Pan et al. have disclosed another TNF receptor family member referred to as "DR4" [Pan et al., Science, 276:111-113 (1997)]. The DR4 was reported to contain a cytoplasmic death domain capable of engaging the cell suicide apparatus. Pan et al. disclose that DR4 is believed to be a receptor for the ligand known as Apo-2 ligand or TRAIL.

#### The Apoptosis-Inducing Signaling Complex

35 As presently understood, the cell death program contains at least three important elements - activators, inhibitors, and effectors; in *C. elegans*, these elements are encoded respectively by three genes, *Ced-4*, *Ced-9* and *Ced-3* [Steller, Science, 267:1445 (1995); Chinnaiyan et al., Science, 275:1122-1126 (1997)]. Two of the TNFR family members,

5 TNFR1 and Fas/Apo1 (CD95), can activate apoptotic cell death [Chinnaiyan and Dixit, Current Biology, 6:555-562 (1996); Fraser and Evan, Cell; 85:781-784 (1996)]. TNFR1 is also known to mediate activation of the transcription factor, NF-KB [Tartaglia et al., Cell, 74:845-853 (1993); Hsu et al., Cell, 84:299-308 (1996)]. In addition to some ECD homology, 10 these two receptors share homology in their intracellular domain (ICD) in an oligomerization interface known as the death domain [Tartaglia et al., supra; Nagata, Cell, 88:355 (1997)]. Death domains are also found in several metazoan proteins that regulate apoptosis, namely, the Drosophila protein, Reaper, and the mammalian proteins referred to as 15 FADD/MORT1, TRADD, and RIP [Cleaveland and Ihle, Cell, 81:479-482 (1995)]. Using the yeast-two hybrid system, Raven et al. report the identification of protein, wsl-1, which binds to the TNFR1 death domain [Raven et al., Programmed Cell Death Meeting, September 20-24, 1995, Abstract at page 127; Raven et al., European Cytokine Network, 7:Abstr. 20 82 at page 210 (April-June 1996)]. The wsl-1 protein is described as being homologous to TNFR1 (48% identity) and having a restricted tissue distribution. According to Raven et al., the tissue distribution of wsl-1 is significantly different from the TNFR1 binding protein, TRADD.

Upon ligand binding and receptor clustering, TNFR1 and CD95 25 are believed to recruit FADD into a death-inducing signalling complex. CD95 purportedly binds FADD directly, while TNFR1 binds FADD indirectly via TRADD [Chinnaiyan et al., Cell, 81:505-512 (1995); Boldin et al., J. Biol. Chem., 270:387-391 (1995); Hsu et al., supra; Chinnaiyan et al., J. Biol. Chem., 271:4961-4965 (1996)]. It has been reported that FADD 30 serves as an adaptor protein which recruits the Ced-3-related protease, MACH $\alpha$ /FLICE (caspase 8), into the death signalling complex [Boldin et al., Cell, 85:803-815 (1996); Muzio et al., Cell, 85:817-827 (1996)]. MACH $\alpha$ /FLICE appears to be the trigger that sets off a cascade of apoptotic proteases, including the interleukin-1 $\beta$  converting enzyme 35 (ICE) and CPP32/Yama, which may execute some critical aspects of the cell death programme [Fraser and Evan, supra].

It was recently disclosed that programmed cell death involves the activity of members of a family of cysteine proteases related to the *C. elegans* cell death gene, *ced-3*, and to the mammalian IL-1-converting 40 enzyme, ICE. The activity of the ICE and CPP32/Yama proteases can be inhibited by the product of the cowpox virus gene, *crmA* [Ray et al.,



5 Cell, 69:597-604 (1992); Tewari et al., Cell, 81:801-809 (1995)]. Recent studies show that CrmA can inhibit TNFR1- and CD95-induced cell death [Enari et al., Nature, 375:78-81 (1995); Tewari et al., J. Biol. Chem., 270:3255-3260 (1995)].

10 As reviewed recently by Tewari et al., TNFR1, TNFR2 and CD40 modulate the expression of proinflammatory and costimulatory cytokines, cytokine receptors, and cell adhesion molecules through activation of the transcription factor, NF-KB [Tewari et al., Curr. Op. Genet. Develop., 6:39-44 (1996)]. NF-KB is the prototype of a family of dimeric transcription factors whose subunits contain conserved Rel regions [Verma et al., Genes Develop., 9:2723-2735 (1996); Baldwin, Ann. Rev. Immunol., 14:649-681 (1996)]. In its latent form, NF-KB is complexed with members of the IKB inhibitor family; upon inactivation of the IKB in response to certain stimuli, released NF-KB translocates to the nucleus where it binds to specific DNA sequences and activates gene transcription.

20 For a review of the TNF family of cytokines and their receptors, see Gruss and Dower, supra.

#### SUMMARY OF THE INVENTION

25 Applicants have identified cDNA clones that encode novel polypeptides, designated in the present application as "Apo-2." It is believed that Apo-2 is a member of the TNFR family; full-length native sequence human Apo-2 polypeptide exhibits some similarities to some known TNFRs, including a cytoplasmic death domain region. Full-length native sequence human Apo-2 also exhibits similarity to the TNFR family in its extracellular cysteine-rich repeats. Apo-2 polypeptide has been found to be capable of triggering caspase-dependent apoptosis and activating NF-KB. Applicants surprisingly found that the soluble extracellular domain of Apo-2 binds Apo-2 ligand ("Apo-2L") and can inhibit Apo-2 ligand function. It is presently believed that Apo-2 ligand can signal via at least two different receptors, DR4 and the newly described Apo-2 herein.

35 In one embodiment, the invention provides isolated Apo-2 polypeptide. In particular, the invention provides isolated native sequence Apo-2 polypeptide, which in one embodiment, includes an amino

40

acid sequence comprising residues 1 to 411 of Figure 1 (SEQ ID NO:1). In other embodiments, the isolated Apo-2 polypeptide comprises at least about 80% amino acid sequence identity with native sequence Apo-2 polypeptide comprising residues 1 to 411 of Figure 1 (SEQ ID NO:1). Optionally, the Apo-2 polypeptide is obtained or obtainable by expressing the polypeptide encoded by the cDNA insert of the vector deposited as ATCC 209021.

In another embodiment, the invention provides an isolated extracellular domain (ECD) sequence of Apo-2. Optionally, the isolated extracellular domain sequence comprises amino acid residues 54 to 182 of Fig. 1 (SEQ ID NO:1).

In another embodiment, the invention provides an isolated death domain sequence of Apo-2. Optionally, the isolated death domain sequence comprises amino acid residues 324 to 391 of Fig. 1 (SEQ ID NO:1).

In another embodiment, the invention provides chimeric molecules comprising Apo-2 polypeptide fused to a heterologous polypeptide or amino acid sequence. An example of such a chimeric molecule comprises an Apo-2 fused to an immunoglobulin sequence. Another example comprises an extracellular domain sequence of Apo-2 fused to a heterologous polypeptide or amino acid sequence, such as an immunoglobulin sequence.

In another embodiment, the invention provides an isolated nucleic acid molecule encoding Apo-2 polypeptide. In one aspect, the nucleic acid molecule is RNA or DNA that encodes an Apo-2 polypeptide or a particular domain of Apo-2, or is complementary to such encoding nucleic acid sequence, and remains stably bound to it under at least moderate, and optionally, under high stringency conditions. In one embodiment, the nucleic acid sequence is selected from:

(a) the coding region of the nucleic acid sequence of Figure 1 (SEQ ID NO:2) that codes for residue 1 to residue 411 (i.e., nucleotides 140-142 through 1370-1372), inclusive;

(b) the coding region of the nucleic acid sequence of Figure 1 (SEQ ID NO:2) that codes for residue 1 to residue 182 (i.e., nucleotides 140-142 through 683-685), inclusive;

(c) the coding region of the nucleic acid sequence of Figure 1 (SEQ ID NO:2) that codes for residue 54 to residue 182 (i.e., nucleotides 299-301 through 683-685), inclusive;

5 (d) the coding region of the nucleic acid sequence of Figure 1 (SEQ ID NO:2) that codes for residue 324 to residue 391 (i.e., nucleotides 1109-1111 through 1310-1312), inclusive; or

(e) a sequence corresponding to the sequence of (a), (b), (c) or (d) within the scope of degeneracy of the genetic code. The isolated  
10 nucleic acid may comprise the Apo-2 polypeptide cDNA insert of the vector deposited as ATCC 209021 which includes the nucleotide sequence encoding Apo-2 polypeptide.

In a further embodiment, the invention provides a vector comprising the nucleic acid molecule encoding the Apo-2 polypeptide or  
15 particular domain of Apo-2. A host cell comprising the vector or the nucleic acid molecule is also provided. A method of producing Apo-2 is further provided.

In another embodiment, the invention provides an antibody which specifically binds to Apo-2. The antibody may be an agonistic, antagonistic or neutralizing antibody. Dimeric molecules, in particular  
20 homodimeric molecules, comprising Apo-2 antibody are also provided.

In another embodiment, the invention provides non-human, transgenic or knock-out animals.

A further embodiment of the invention provides articles of  
25 manufacture and kits that include Apo-2 or Apo-2 antibodies.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the nucleotide sequence of a native sequence human Apo-2 cDNA and its derived amino acid sequence.

30 Figure 2A shows the derived amino acid sequence of a native sequence human Apo-2 - the putative signal sequence is underlined, the putative transmembrane domain is boxed, and the putative death domain sequence is dash underlined. The cysteines of the two cysteine-rich domains are individually underlined.

35 Figure 2B shows an alignment and comparison of the death domain sequences of native sequence human Apo-2, DR4, Apo-3/DR3, TNFR1, and Fas/Apo-1 (CD95). Asterisks indicate residues that are essential for death signaling by TNFR1 [Tartaglia et al., supra].

40 Figure 3 shows the interaction of the Apo-2 ECD with Apo-2L. Supernatants from mock-transfected 293 cells or from 293 cells transfected with Flag epitope-tagged Apo-2 ECD were incubated with poly-His-tagged Apo-2L and subjected to immunoprecipitation with anti-Flag

5 conjugated or Nickel conjugated agarose beads. The precipitated proteins were resolved by electrophoresis on polyacrylamide gels, and detected by immunoblot with anti-Apo-2L or anti-Flag antibody.

10 Figure 4 shows the induction of apoptosis by Apo-2 and inhibition of Apo-2L activity by soluble Apo-2 ECD. Human 293 cells (A, B) or HeLa cells (C) were transfected by pRK5 vector or by pRK5-based plasmids encoding Apo-2 and/or CrmA. Apoptosis was assessed by morphology (A), DNA fragmentation (B), or by FACS (C-E). Soluble Apo-2L was pre-incubated with buffer or affinity-purified Apo-2 ECD together with anti-Flag antibody or Apo-2 ECD immunoaderhin or DR4 or TNFR1  
15 immunoaderhins and added to HeLa cells. The cells were later analyzed for apoptosis (D). Dose-response analysis using Apo-2L with Apo-2 ECD immunoaderhin was also determined (E).

20 Figure 5 shows activation of NF-KB by Apo-2, DR4, and Apo-2L. (A) HeLa cells were transfected with expression plasmids encoding the indicated proteins. Nuclear extracts were prepared and analyzed by an electrophoretic mobility shift assay. (B) HeLa cells or MCF7 cells were treated with buffer, Apo-2L or TNF-alpha and assayed for NF-KB activity. (C) HeLa cells were preincubated with buffer, ALLN or cyclohexamide before addition of Apo-2L. Apoptosis was later analyzed by FACS.

25 Figure 6 shows expression of Apo-2 mRNA in human tissues as analyzed by Northern hybridization of human tissue poly A RNA blots.

30 Figure 7 shows the FACS analysis of an Apo-2 antibody, 3F11.39.7 (illustrated by the bold lines) as compared to IgG controls (dotted lines). The 3F11.39.7 antibody recognized the Apo-2 receptor expressed in human 9D cells.

35 Figure 8 is a graph showing percent (%) apoptosis induced in 9D cells by Apo-2 antibody 3F11.39.7, in the absence of goat anti-mouse IgG Fc.

Figure 9 is a bar diagram showing percent (%) apoptosis, as compared to Apo-2L, in 9D cells by Apo-2 antibody 3F11.39.7 in the presence or absence of goat anti-mouse IgG Fc.

Figure 10 is a bar diagram illustrating the ability of Apo-2 antibody 3F11.39.7 to block the apoptosis induced by Apo-2L in 9D cells.

40 Figure 11 is a graph showing results of an ELISA testing binding of Apo-2 antibody 3F11.39.7 to Apo-2 and to other known Apo-2L

5 receptors referred to as DR4, DcR1, and DcR2.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

##### I. Definitions

10 The terms "Apo-2 polypeptide" and "Apo-2" when used herein encompass native sequence Apo-2 and Apo-2 variants (which are further defined herein). These terms encompass Apo-2 from a variety of mammals, including humans. The Apo-2 may be isolated from a variety of sources, such as from human tissue types or from another source, or prepared by  
15 recombinant or synthetic methods.

A "native sequence Apo-2" comprises a polypeptide having the same amino acid sequence as an Apo-2 derived from nature. Thus, a native sequence Apo-2 can have the amino acid sequence of naturally-occurring Apo-2 from any mammal. Such native sequence Apo-2 can be  
20 isolated from nature or can be produced by recombinant or synthetic means. The term "native sequence Apo-2" specifically encompasses naturally-occurring truncated or secreted forms of the Apo-2 (e.g., an extracellular domain sequence), naturally-occurring variant forms (e.g., alternatively spliced forms) and naturally-occurring allelic variants of the Apo-2. A naturally-occurring variant form of the Apo-2 includes an Apo-2 having an amino acid substitution at residue 410 in the amino acid sequence shown in Figure 1 (SEQ ID NO:1). In one embodiment of such naturally-occurring variant form, the leucine residue at position 410 is substituted by a methionine residue. In Fig. 1 (SEQ ID NO:1), the amino  
25 acid residue at position 410 is identified as "Xaa" to indicate that the amino acid may, optionally, be either leucine or methionine. In Fig. 1 (SEQ ID NO:2), the nucleotide at position 1367 is identified as "W" to indicate that the nucleotide may be either adenine (A) or thymine (T) or uracil (U). In one embodiment of the invention, the native sequence  
30 Apo-2 is a mature or full-length native sequence Apo-2 comprising amino acids 1 to 411 of Fig. 1 (SEQ ID NO:1).

The "Apo-2 extracellular domain" or "Apo-2 ECD" refers to a form of Apo-2 which is essentially free of the transmembrane and cytoplasmic domains of Apo-2. Ordinarily, Apo-2 ECD will have less than  
40 1% of such transmembrane and/or cytoplasmic domains and preferably, will have less than 0.5% of such domains. Optionally, Apo-2 ECD will comprise amino acid residues 54 to 182 of Fig. 1 (SEQ ID NO:1) or amino

5 acid residues 1 to 182 of Fig. 1 (SEQ ID NO:1).

"Apo-2 variant" means a biologically active Apo-2 as defined below having at least about 80% amino acid sequence identity with the Apo-2 having the deduced amino acid sequence shown in Fig. 1 (SEQ ID NO:1) for a full-length native sequence human Apo-2. Such Apo-2 variants include, for instance, Apo-2 polypeptides wherein one or more amino acid residues are added, or deleted, at the N- or C-terminus of the sequence of Fig. 1 (SEQ ID NO:1). Ordinarily, an Apo-2 variant will have at least about 80% amino acid sequence identity, more preferably at least about 90% amino acid sequence identity, and even more preferably at least about 95% amino acid sequence identity with the amino acid sequence of Fig. 1 (SEQ ID NO:1).

"Percent (%) amino acid sequence identity" with respect to the Apo-2 sequences identified herein is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the Apo-2 sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as ALIGN™ or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared.

The term "epitope tagged" when used herein refers to a chimeric polypeptide comprising Apo-2, or a domain sequence thereof, fused to a "tag polypeptide". The tag polypeptide has enough residues to provide an epitope against which an antibody can be made, yet is short enough such that it does not interfere with activity of the Apo-2. The tag polypeptide preferably also is fairly unique so that the antibody does not substantially cross-react with other epitopes. Suitable tag polypeptides generally have at least six amino acid residues and usually between about 8 to about 50 amino acid residues (preferably, between about 10 to about 20 residues).

"Isolated," when used to describe the various polypeptides disclosed herein, means polypeptide that has been identified and separated and/or recovered from a component of its natural environment.

5 Contaminant components of its natural environment are materials that would typically interfere with diagnostic or therapeutic uses for the polypeptide, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In preferred embodiments, the polypeptide will be purified (1) to a degree sufficient to obtain at least 15  
10 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (2) to homogeneity by SDS-PAGE under non-reducing or reducing conditions using Coomassie blue or, preferably, silver stain. Isolated polypeptide includes polypeptide *in situ* within recombinant cells, since at least one component of the Apo-2 natural  
15 environment will not be present. Ordinarily, however, isolated polypeptide will be prepared by at least one purification step.

An "isolated" Apo-2 nucleic acid molecule is a nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the  
20 natural source of the Apo-2 nucleic acid. An isolated Apo-2 nucleic acid molecule is other than in the form or setting in which it is found in nature. Isolated Apo-2 nucleic acid molecules therefore are distinguished from the Apo-2 nucleic acid molecule as it exists in natural cells. However, an isolated Apo-2 nucleic acid molecule  
25 includes Apo-2 nucleic acid molecules contained in cells that ordinarily express Apo-2 where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a  
30 particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a  
35 functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the  
40 sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked

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are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

The term "antibody" is used in the broadest sense and specifically covers anti-Apo-2 monoclonal antibodies (including agonist, antagonist, and blocking or neutralizing antibodies) and anti-Apo-2 antibody compositions with polyepitopic specificity.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally-occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen.

The monoclonal antibodies herein include hybrid and recombinant antibodies produced by splicing a variable (including hypervariable) domain of an anti-Apo-2 antibody with a constant domain, or a light chain with a heavy chain, or a chain from one species with a chain from another species, or fusions with heterologous proteins, regardless of species of origin or immunoglobulin class or subclass designation, as well as antibody fragments (e.g., Fab, F(ab')<sub>2</sub>, and Fv), so long as they exhibit the desired biological activity. See, e.g. U.S. Pat. No. 4,816,567 and Mage et al., in Monoclonal Antibody Production Techniques and Applications, pp.79-97 (Marcel Dekker, Inc.: New York, 1987).

Thus, the modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler and Milstein, Nature, 256:495 (1975), or may be made by recombinant DNA methods such as described in U.S. Pat. No. 4,816,567. The "monoclonal



5 antibodies" may also be isolated from phage libraries generated using  
the techniques described in McCafferty et al., Nature, 348:552-554  
(1990), for example.

10 "Humanized" forms of non-human (e.g. murine) antibodies are  
specific chimeric immunoglobulins, immunoglobulin chains, or fragments  
thereof (such as Fv, Fab, Fab', F(ab')<sub>2</sub> or other antigen-binding  
subsequences of antibodies) which contain minimal sequence derived from  
non-human immunoglobulin. For the most part, humanized antibodies are  
human immunoglobulins (recipient antibody) in which residues from a  
complementary determining region (CDR) of the recipient are replaced by  
15 residues from a CDR of a non-human species (donor antibody) such as  
mouse, rat, or rabbit having the desired specificity, affinity, and  
capacity. In some instances, Fv framework region (FR) residues of the  
human immunoglobulin are replaced by corresponding non-human residues.  
Furthermore, the humanized antibody may comprise residues which are  
20 found neither in the recipient antibody nor in the imported CDR or  
framework sequences. These modifications are made to further refine and  
optimize antibody performance. In general, the humanized antibody will  
comprise substantially all of at least one, and typically two, variable  
domains, in which all or substantially all of the CDR regions correspond  
25 to those of a non-human immunoglobulin and all or substantially all of  
the FR regions are those of a human immunoglobulin consensus sequence.  
The humanized antibody optimally also will comprise at least a portion  
of an immunoglobulin constant region or domain (Fc), typically that of a  
human immunoglobulin.

30 "Biologically active" and "desired biological activity" for  
the purposes herein mean having the ability to modulate apoptosis  
(either in an agonistic or stimulating manner or in an antagonistic or  
blocking manner) in at least one type of mammalian cell *in vivo* or *ex*  
*vivo*.

35 The terms "apoptosis" and "apoptotic activity" are used in a  
broad sense and refer to the orderly or controlled form of cell death in  
mammals that is typically accompanied by one or more characteristic cell  
changes, including condensation of cytoplasm, loss of plasma membrane  
microvilli, segmentation of the nucleus, degradation of chromosomal DNA  
40 or loss of mitochondrial function. This activity can be determined and  
measured, for instance, by cell viability assays, FACS analysis or DNA  
electrophoresis, all of which are known in the art.

5 The terms "treating," "treatment," and "therapy" as used herein refer to curative therapy, prophylactic therapy, and preventative therapy.

The term "mammal" as used herein refers to any mammal classified as a mammal, including humans, cows, horses, dogs and cats.

10 In a preferred embodiment of the invention, the mammal is a human.

## II. Compositions and Methods of the Invention

The present invention provides newly identified and isolated Apo-2 polypeptides and Apo-2 antibodies. In particular, Applicants have identified and isolated various human Apo-2 polypeptides. The properties and characteristics of some of these Apo-2 polypeptides and anti-Apo-2 antibodies are described in further detail in the Examples below. Based upon the properties and characteristics of the Apo-2 polypeptides disclosed herein, it is Applicants' present belief that Apo-2 is a member of the TNFR family.

A description follows as to how Apo-2, as well as Apo-2 chimeric molecules and anti-Apo-2 antibodies, may be prepared.

### A. Preparation of Apo-2

The description below relates primarily to production of Apo-2 by culturing cells transformed or transfected with a vector containing Apo-2 nucleic acid. It is of course, contemplated that alternative methods, which are well known in the art, may be employed to prepare Apo-2.

#### 1. Isolation of DNA Encoding Apo-2

The DNA encoding Apo-2 may be obtained from any cDNA library prepared from tissue believed to possess the Apo-2 mRNA and to express it at a detectable level. Accordingly, human Apo-2 DNA can be conveniently obtained from a cDNA library prepared from human tissues, such as the bacteriophage libraries of human pancreas and kidney cDNA described in Example 1. The Apo-2-encoding gene may also be obtained from a genomic library or by oligonucleotide synthesis.

Libraries can be screened with probes (such as antibodies to the Apo-2 or oligonucleotides of at least about 20-80 bases) designed to identify the gene of interest or the protein encoded by it. Screening the cDNA or genomic library with the selected probe may be conducted using standard procedures, such as described in Sambrook et al.,

5 Molecular Cloning: A Laboratory Manual (New York: Cold Spring Harbor Laboratory Press, 1989). An alternative means to isolate the gene encoding Apo-2 is to use PCR methodology [Sambrook et al., supra; Dieffenbach et al., PCR Primer: A Laboratory Manual (Cold Spring Harbor Laboratory Press, 1995)].

10 A preferred method of screening employs selected oligonucleotide sequences to screen cDNA libraries from various human tissues. Example 1 below describes techniques for screening a cDNA library. The oligonucleotide sequences selected as probes should be of sufficient length and sufficiently unambiguous that false positives are  
15 minimized. The oligonucleotide is preferably labeled such that it can be detected upon hybridization to DNA in the library being screened. Methods of labeling are well known in the art, and include the use of radiolabels like <sup>32</sup>P-labeled ATP, biotinylation or enzyme labeling. Hybridization conditions, including moderate stringency and high  
20 stringency, are provided in Sambrook et al., supra.

Nucleic acid having all the protein coding sequence may be obtained by screening selected cDNA or genomic libraries using the deduced amino acid sequence disclosed herein for the first time, and, if necessary, using conventional primer extension procedures as described  
25 in Sambrook et al., supra, to detect precursors and processing intermediates of mRNA that may not have been reverse-transcribed into cDNA.

Apo-2 variants can be prepared by introducing appropriate nucleotide changes into the Apo-2 DNA, or by synthesis of the desired  
30 Apo-2 polypeptide. Those skilled in the art will appreciate that amino acid changes may alter post-translational processes of the Apo-2, such as changing the number or position of glycosylation sites or altering the membrane anchoring characteristics.

Variations in the native full-length sequence Apo-2 or in  
35 various domains of the Apo-2 described herein, can be made, for example, using any of the techniques and guidelines for conservative and non-conservative mutations set forth, for instance, in U.S. Pat. No. 5,364,934. Variations may be a substitution, deletion or insertion of one or more codons encoding the Apo-2 that results in a change in the  
40 amino acid sequence of the Apo-2 as compared with the native sequence Apo-2. Optionally the variation is by substitution of at least one amino acid with any other amino acid in one or more of the domains of

5 the Apo-2 molecule. The variations can be made using methods known in  
the art such as oligonucleotide-mediated (site-directed) mutagenesis,  
alanine scanning, and PCR mutagenesis. Site-directed mutagenesis  
[Carter et al., Nucl. Acids Res., 13:4331 (1986); Zoller et al., Nucl.  
Acids Res., 10:6487 (1987)], cassette mutagenesis [Wells et al., Gene,  
10 34:315 (1985)], restriction selection mutagenesis [Wells et al., Philos.  
Trans. R. Soc. London SerA, 317:415 (1986)] or other known techniques  
can be performed on the cloned DNA to produce the Apo-2 variant DNA.

Scanning amino acid analysis can also be employed to identify  
one or more amino acids along a contiguous sequence which are involved  
15 in the interaction with a particular ligand or receptor. Among the  
preferred scanning amino acids are relatively small, neutral amino  
acids. Such amino acids include alanine, glycine, serine, and cysteine.  
Alanine is the preferred scanning amino acid among this group because it  
eliminates the side-chain beyond the beta-carbon and is less likely to  
20 alter the main-chain conformation of the variant. Alanine is also  
preferred because it is the most common amino acid. Further, it is  
frequently found in both buried and exposed positions [Creighton, The  
Proteins, (W.H. Freeman & Co., N.Y.); Chothia, J. Mol. Biol., 150:1  
(1976)]. If alanine substitution does not yield adequate amounts of  
25 variant, an isoteric amino acid can be used.

Once selected Apo-2 variants are produced, they can be  
contacted with, for instance, Apo-2L, and the interaction, if any, can  
be determined. The interaction between the Apo-2 variant and Apo-2L can  
be measured by an *in vitro* assay, such as described in the Examples  
30 below. While any number of analytical measurements can be used to  
compare activities and properties between a native sequence Apo-2 and an  
Apo-2 variant, a convenient one for binding is the dissociation constant  
 $K_d$  of the complex formed between the Apo-2 variant and Apo-2L as  
compared to the  $K_d$  for the native sequence Apo-2. Generally, a  $\geq 3$ -fold  
35 increase or decrease in  $K_d$  per substituted residue indicates that the  
substituted residue(s) is active in the interaction of the native  
sequence Apo-2 with the Apo-2L.

Optionally, representative sites in the Apo-2 sequence  
suitable for mutagenesis would include sites within the extracellular  
40 domain, and particularly, within one or both of the cysteine-rich  
domains. Such variations can be accomplished using the methods  
described above.

5                   2.   Insertion of Nucleic Acid into A Replicable Vector

The nucleic acid (e.g., cDNA or genomic DNA) encoding Apo-2 may be inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. Various vectors are publicly available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence, each of which is described below.

                  (i)   Signal Sequence Component

15                   The Apo-2 may be produced recombinantly not only directly, but also as a fusion polypeptide with a heterologous polypeptide, which may be a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. In general, the signal sequence may be a component of the vector, or it may be a part of the Apo-2 DNA that is inserted into the vector. The heterologous signal sequence selected preferably is one that is recognized and processed (i.e., cleaved by a signal peptidase) by the host cell. The signal sequence may be a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion the signal sequence may be, e.g., the yeast invertase leader, alpha factor leader (including *Saccharomyces* and *Kluyveromyces*  $\alpha$ -factor leaders, the latter described in U.S. Pat. No. 5,010,182), or acid phosphatase leader, the *C. albicans* glucoamylase leader (EP 362,179 published 4 April 1990), or the signal described in WO 90/13646 published 15 November 1990. In mammalian cell expression the native Apo-2 presequence that normally directs insertion of Apo-2 in the cell membrane of human cells *in vivo* is satisfactory, although other mammalian signal sequences may be used to direct secretion of the protein, such as signal sequences from secreted polypeptides of the same or related species, as well as viral secretory leaders, for example, the herpes simplex glycoprotein D signal.

                  The DNA for such precursor region is preferably ligated in reading frame to DNA encoding Apo-2.

40                   (ii)   Origin of Replication Component

Both expression and cloning vectors contain a nucleic acid

5 sequence that enables the vector to replicate in one or more selected  
host cells. Generally, in cloning vectors this sequence is one that  
enables the vector to replicate independently of the host chromosomal  
DNA, and includes origins of replication or autonomously replicating  
10 sequences. Such sequences are well known for a variety of bacteria,  
yeast, and viruses. The origin of replication from the plasmid pBR322  
is suitable for most Gram-negative bacteria, the 2 $\mu$  plasmid origin is  
suitable for yeast, and various viral origins (SV40, polyoma,  
adenovirus, VSV or BPV) are useful for cloning vectors in mammalian  
15 cells. Generally, the origin of replication component is not needed for  
mammalian expression vectors (the SV40 origin may typically be used  
because it contains the early promoter).

Most expression vectors are "shuttle" vectors, i.e., they are  
capable of replication in at least one class of organisms but can be  
transfected into another organism for expression. For example, a vector  
20 is cloned in *E. coli* and then the same vector is transfected into yeast  
or mammalian cells for expression even though it is not capable of  
replicating independently of the host cell chromosome.

DNA may also be amplified by insertion into the host genome.  
This is readily accomplished using *Bacillus* species as hosts, for  
25 example, by including in the vector a DNA sequence that is complementary  
to a sequence found in *Bacillus* genomic DNA. Transfection of *Bacillus*  
with this vector results in homologous recombination with the genome and  
insertion of Apo-2 DNA. However, the recovery of genomic DNA encoding  
Apo-2 is more complex than that of an exogenously replicated vector  
30 because restriction enzyme digestion is required to excise the Apo-2  
DNA.

### (iii) Selection Gene Component

Expression and cloning vectors typically contain a selection  
gene, also termed a selectable marker. This gene encodes a protein  
35 necessary for the survival or growth of transformed host cells grown in  
a selective culture medium. Host cells not transformed with the vector  
containing the selection gene will not survive in the culture medium.  
Typical selection genes encode proteins that (a) confer resistance to  
antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate,  
40 or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply  
critical nutrients not available from complex media, e.g., the gene

5 encoding D-alanine racemase for *Bacilli*.

One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene produce a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant  
10 selection use the drugs neomycin [Southern et al., J. Molec. Appl. Genet., 1:327 (1982)], mycophenolic acid (Mulligan et al., Science, 209:1422 (1980)] or hygromycin [Sugden et al., Mol. Cell. Biol., 5:410-413 (1985)]. The three examples given above employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug  
15 G418 or neomycin (geneticin), xgpt (mycophenolic acid), or hygromycin, respectively.

Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the Apo-2 nucleic acid, such as DHFR or thymidine kinase. The mammalian cell transformants are placed under selection pressure that only the transformants are uniquely adapted to survive by virtue of having taken up the marker. Selection pressure is imposed by culturing the transformants under conditions in which the concentration of selection agent in the medium is successively changed, thereby leading  
20 to amplification of both the selection gene and the DNA that encodes Apo-2. Amplification is the process by which genes in greater demand for the production of a protein critical for growth are reiterated in tandem within the chromosomes of successive generations of recombinant cells. Increased quantities of Apo-2 are synthesized from the amplified  
25 DNA. Other examples of amplifiable genes include metallothionein-I and -II, adenosine deaminase, and ornithine decarboxylase.  
30

Cells transformed with the DHFR selection gene may first be identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An  
35 appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub et al., Proc. Natl. Acad. Sci. USA, 77:4216 (1980). The transformed cells are then exposed to increased levels of methotrexate. This leads to the synthesis of multiple copies  
40 of the DHFR gene, and, concomitantly, multiple copies of other DNA comprising the expression vectors, such as the DNA encoding Apo-2. This amplification technique can be used with any otherwise suitable host,

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5 e.g., ATCC No. CCL61 CHO-K1, notwithstanding the presence of endogenous DHFR if, for example, a mutant DHFR gene that is highly resistant to Mtx is employed (EP 117,060).

10 Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding Apo-2, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3'-phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, e.g., kanamycin, neomycin, or G418. See U.S. Patent No. 4,965,199.

15 A suitable selection gene for use in yeast is the *trp1* gene present in the yeast plasmid YRp7 [Stinchcomb et al., Nature, 282:39 (1979); Kingsman et al., Gene, 7:141 (1979); Tschemper et al., Gene, 10:157 (1980)]. The *trp1* gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1 [Jones, Genetics, 85:12 (1977)]. The presence of the *trp1* lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, *Leu2*-deficient yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the *Leu2* gene.

25 In addition, vectors derived from the 1.6  $\mu$ m circular plasmid pKD1 can be used for transformation of *Kluyveromyces* yeasts [Bianchi et al., Curr. Genet., 12:185 (1987)]. More recently, an expression system for large-scale production of recombinant calf chymosin was reported for *K. lactis* [Van den Berg, Bio/Technology, 8:135 (1990)]. Stable multi-copy expression vectors for secretion of mature recombinant human serum albumin by industrial strains of *Kluyveromyces* have also been disclosed [Fleer et al., Bio/Technology, 9:968-975 (1991)].

(iv) Promoter Component

35 Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the Apo-2 nucleic acid sequence. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of particular nucleic acid sequence, such as the Apo-2 nucleic acid sequence, to which they are operably linked. Such



5 promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, e.g., the presence or absence of a nutrient or a change in temperature. At this time a large number of promoters  
10 recognized by a variety of potential host cells are well known. These promoters are operably linked to Apo-2 encoding DNA by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector. Both the native Apo-2 promoter sequence and many heterologous promoters may be  
15 used to direct amplification and/or expression of the Apo-2 DNA.

Promoters suitable for use with prokaryotic hosts include the  $\beta$ -lactamase and lactose promoter systems [Chang et al., Nature, 275:615 (1978); Goeddel et al., Nature, 281:544 (1979)], alkaline phosphatase, a tryptophan (trp) promoter system [Goeddel, Nucleic Acids Res., 8:4057 (1980); EP 36,776], and hybrid promoters such as the tac promoter [deBoer et al., Proc. Natl. Acad. Sci. USA, 80:21-25 (1983)]. However,  
20 other known bacterial promoters are suitable. Their nucleotide sequences have been published, thereby enabling a skilled worker operably to ligate them to DNA encoding Apo-2 [Siebenlist et al., Cell, 20:269 (1980)] using linkers or adaptors to supply any required  
25 restriction sites. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding Apo-2.

Promoter sequences are known for eukaryotes. Virtually all  
30 eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CXCAAT region where X may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the  
35 signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into eukaryotic expression vectors.

Examples of suitable promoting sequences for use with yeast  
40 hosts include the promoters for 3-phosphoglycerate kinase [Hitzeman et al., J. Biol. Chem., 255:2073 (1980)] or other glycolytic enzymes [Hess et al., J. Adv. Enzyme Req., 7:149 (1968); Holland, Biochemistry,

5 17:4900 (1978)], such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

10 Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate  
15 dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in EP 73,657. Yeast enhancers also are advantageously used with yeast promoters.

20 Apo-2 transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published 5 July 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40), from heterologous mammalian  
25 promoters, e.g., the actin promoter or an immunoglobulin promoter, from heat-shock promoters, and from the promoter normally associated with the Apo-2 sequence, provided such promoters are compatible with the host cell systems.

30 The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication [Fiers et al., Nature, 273:113 (1978); Mulligan and Berg, Science, 209:1422-1427 (1980); Pavlakis et al., Proc. Natl. Acad. Sci. USA, 78:7398-7402 (1981)]. The immediate early promoter of the human cytomegalovirus is conveniently obtained as  
35 a HindIII E restriction fragment [Greenaway et al., Gene, 18:355-360 (1982)]. A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. Patent No. 4,419,446. A modification of this system is described in U.S. Patent No. 4,601,978 [See also Gray et al., Nature, 295:503-508 (1982) on expressing cDNA  
40 encoding immune interferon in monkey cells; Reyes et al., Nature, 297:598-601 (1982) on expression of human  $\beta$ -interferon cDNA in mouse

5 cells under the control of a thymidine kinase promoter from herpes simplex virus; Canaani and Berg, Proc. Natl. Acad. Sci. USA 79:5166-5170 (1982) on expression of the human interferon gene in cultured mouse and rabbit cells; and Gorman et al., Proc. Natl. Acad. Sci. USA, 79:6777-6781 (1982) on expression of bacterial CAT sequences in CV-1 monkey  
 10 kidney cells, chicken embryo fibroblasts, Chinese hamster ovary cells, HeLa cells, and mouse NIH-3T3 cells using the Rous sarcoma virus long terminal repeat as a promoter].

(v) Enhancer Element Component

Transcription of a DNA encoding the Apo-2 of this invention  
 15 by higher eukaryotes may be increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp, that act on a promoter to increase its transcription. Enhancers are relatively orientation and position independent, having been found 5' [Laimins et al., Proc. Natl. Acad. Sci. USA, 78:993 (1981)] and 3' [Lusky et al., Mol. Cell Bio., 3:1108 (1983)] to the transcription unit, within an intron [Banerji et al., Cell, 33:729 (1983)], as well as within the coding sequence itself [Osborne et al., Mol. Cell Bio., 4:1293 (1984)]. Many enhancer  
 20 sequences are now known from mammalian genes (globin, elastase, albumin, α-fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also  
 25 Yaniv, Nature, 297:17-18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to the Apo-2 coding sequence, but is preferably located at a site 5' from the promoter.

(vi) Transcription Termination Component

35 Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3',  
 40 untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated

5 fragments in the untranslated portion of the mRNA encoding Apo-2.

(vii) Construction and Analysis of Vectors

Construction of suitable vectors containing one or more of the above-listed components employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and re-ligated  
10 in the form desired to generate the plasmids required.

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures can be used to transform *E. coli* K12 strain 294 (ATCC 31,446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from  
15 the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced by the method of Messing et al., Nucleic Acids Res., 9:309 (1981) or by the method of Maxim et al., Methods in Enzymology, 65:499 (1980).

(viii) Transient Expression Vectors

Expression vectors that provide for the transient expression  
20 in mammalian cells of DNA encoding Apo-2 may be employed. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn,  
25 synthesizes high levels of a desired polypeptide encoded by the expression vector [Sambrook et al., *supra*]. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of such polypeptides for  
30 desired biological or physiological properties. Thus, transient expression systems are particularly useful in the invention for purposes of identifying Apo-2 variants.

(ix) Suitable Exemplary Vertebrate Cell Vectors

Other methods, vectors, and host cells suitable for  
35 adaptation to the synthesis of Apo-2 in recombinant vertebrate cell culture are described in Gething et al., Nature, 293:620-625 (1981); Mantei et al., Nature, 281:40-46 (1979); EP 117,060; and EP 117,058.

3. Selection and Transformation of Host Cells

Suitable host cells for cloning or expressing the DNA in the  
40 vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include but are not limited to eubacteria, such as Gram-negative or Gram-positive

organisms, for example, Enterobacteriaceae such as *Escherichia*, e.g., *E. coli*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Proteus*, *Salmonella*, e.g., *Salmonella typhimurium*, *Serratia*, e.g., *Serratia marcescans*, and *Shigella*, as well as *Bacilli* such as *B. subtilis* and *B. licheniformis* (e.g., *B. licheniformis* 41P disclosed in DD 266,710 published 12 April 1989), *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*. Preferably, the host cell should secrete minimal amounts of proteolytic enzymes.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for Apo-2-encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein.

Suitable host cells for the expression of glycosylated Apo-2 are derived from multicellular organisms. Such host cells are capable of complex processing and glycosylation activities. In principle, any higher eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified [See, e.g., Luckow et al., *Bio/Technology*, 6:47-55 (1988); Miller et al., in *Genetic Engineering*, Setlow et al., eds., Vol. 8 (Plenum Publishing, 1986), pp. 277-279; and Maeda et al., *Nature*, 315:592-594 (1985)]. A variety of viral strains for transfection are publicly available, e.g., the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV.

Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can be utilized as hosts. Typically, plant cells are transfected by incubation with certain strains of the bacterium *Agrobacterium tumefaciens*. During incubation of the plant cell culture with *A. tumefaciens*, the DNA encoding the Apo-2 can be transferred to the plant cell host such that it is transfected, and will, under appropriate conditions, express the Apo-2-encoding DNA. In addition, regulatory and signal sequences compatible with plant cells are available, such as the nopaline synthase promoter and polyadenylation signal sequences [Depicker et al., *J. Mol. Appl. Gen.*,

5 1:561 (1982)]. In addition, DNA segments isolated from the upstream region of the T-DNA 780 gene are capable of activating or increasing transcription levels of plant-expressible genes in recombinant DNA-containing plant tissue [EP 321,196 published 21 June 1989].

10 Propagation of vertebrate cells in culture (tissue culture) is also well known in the art [See, e.g., Tissue Culture, Academic Press, Kruse and Patterson, editors (1973)]. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., J. Gen  
15 Virol., 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, Proc. Natl. Acad. Sci. USA, 77:4216 (1980)); mouse sertoli cells (TM4, Mather, Biol. Reprod., 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human  
20 cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., Annals N.Y. Acad. Sci., 383:44-68 (1982)); MRC 5 cells; and FS4 cells.

25 Host cells are transfected and preferably transformed with the above-described expression or cloning vectors for Apo-2 production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

30 Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, CaPO<sub>4</sub> and electroporation. Successful transfection is generally recognized when any indication of the  
35 operation of this vector occurs within the host cell.

Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The  
40 calcium treatment employing calcium chloride, as described in Sambrook et al., supra, or electroporation is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. Infection with

5 *Agrobacterium tumefaciens* is used for transformation of certain plant cells, as described by Shaw et al., Gene, 23:315 (1983) and WO 89/05859 published 29 June 1989. In addition, plants may be transfected using ultrasound treatment as described in WO 91/00358 published 10 January 1991.

10 For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb, Virology, 52:456-457 (1978) is preferred. General aspects of mammalian cell host system transformations have been described in U.S. Pat. No. 4,399,216. Transformations into yeast are typically carried out according to the  
15 method of Van Solingen et al., J. Bact., 130:946 (1977) and Hsiao et al., Proc. Natl. Acad. Sci. (USA), 76:3829 (1979). However, other methods for introducing DNA into cells, such as by nuclear microinjection, electroporation, bacterial protoplast fusion with intact cells, or polycations, e.g., polybrene, polyornithine, may also be used.  
20 For various techniques for transforming mammalian cells, see Keown et al., Methods in Enzymology, 185:527-537 (1990) and Mansour et al., Nature, 336:348-352 (1988).

#### 4. Culturing the Host Cells

25 Prokaryotic cells used to produce Apo-2 may be cultured in suitable media as described generally in Sambrook et al., supra.

The mammalian host cells used to produce Apo-2 may be cultured in a variety of media. Examples of commercially available media include Ham's F10 (Sigma), Minimal Essential Medium ("MEM", Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium  
30 ("DMEM", Sigma). Any such media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin™ drug), trace  
35 elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are  
40 those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

In general, principles, protocols, and practical techniques

5 for maximizing the productivity of mammalian cell cultures can be found  
in Mammalian Cell Biotechnology: a Practical Approach, M. Butler, ed.  
(IRL Press, 1991).

The host cells referred to in this disclosure encompass cells  
in culture as well as cells that are within a host animal.

10 5. Detecting Gene Amplification/Expression

Gene amplification and/or expression may be measured in a  
sample directly, for example, by conventional Southern blotting,  
Northern blotting to quantitate the transcription of mRNA [Thomas, Proc.  
Natl. Acad. Sci. USA, 77:5201-5205 (1980)], dot blotting (DNA analysis),  
15 or *in situ* hybridization, using an appropriately labeled probe, based on  
the sequences provided herein. Various labels may be employed, most  
commonly radioisotopes, and particularly <sup>32</sup>P. However, other techniques  
may also be employed, such as using biotin-modified nucleotides for  
introduction into a polynucleotide. The biotin then serves as the site  
20 for binding to avidin or antibodies, which may be labeled with a wide  
variety of labels, such as radionucleotides, fluorescers or enzymes.  
Alternatively, antibodies may be employed that can recognize specific  
duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid  
duplexes or DNA-protein duplexes. The antibodies in turn may be labeled  
25 and the assay may be carried out where the duplex is bound to a surface,  
so that upon the formation of duplex on the surface, the presence of  
antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by  
immunological methods, such as immunohistochemical staining of cells or  
30 tissue sections and assay of cell culture or body fluids, to quantitate  
directly the expression of gene product. With immunohistochemical  
staining techniques, a cell sample is prepared, typically by dehydration  
and fixation, followed by reaction with labeled antibodies specific for  
the gene product coupled, where the labels are usually visually  
35 detectable, such as enzymatic labels, fluorescent labels, or luminescent  
labels.

Antibodies useful for immunohistochemical staining and/or  
assay of sample fluids may be either monoclonal or polyclonal, and may  
be prepared in any mammal. Conveniently, the antibodies may be prepared  
40 against a native sequence Apo-2 polypeptide or against a synthetic  
peptide based on the DNA sequences provided herein or against exogenous  
sequence fused to Apo-2 DNA and encoding a specific antibody epitope.



5                   6.   Purification of Apo-2 Polypeptide

Forms of Apo-2 may be recovered from culture medium or from host cell lysates. If the Apo-2 is membrane-bound, it can be released from the membrane using a suitable detergent solution (e.g. Triton-X 100) or its extracellular domain may be released by enzymatic cleavage.

10                   When Apo-2 is produced in a recombinant cell other than one of human origin, the Apo-2 is free of proteins or polypeptides of human origin. However, it may be desired to purify Apo-2 from recombinant cell proteins or polypeptides to obtain preparations that are substantially homogeneous as to Apo-2. As a first step, the culture  
15 medium or lysate may be centrifuged to remove particulate cell debris. Apo-2 thereafter is purified from contaminant soluble proteins and polypeptides, with the following procedures being exemplary of suitable purification procedures: by fractionation on an ion-exchange column; ethanol precipitation; reverse phase HPLC; chromatography on silica or  
20 on a cation-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; and protein A Sepharose columns to remove contaminants such as IgG.

Apo-2 variants in which residues have been deleted, inserted,  
25 or substituted can be recovered in the same fashion as native sequence Apo-2, taking account of changes in properties occasioned by the variation. For example, preparation of an Apo-2 fusion with another protein or polypeptide, e.g., a bacterial or viral antigen, immunoglobulin sequence, or receptor sequence, may facilitate  
30 purification; an immunoaffinity column containing antibody to the sequence can be used to adsorb the fusion polypeptide. Other types of affinity matrices also can be used.

A protease inhibitor such as phenyl methyl sulfonyl fluoride (PMSF) also may be useful to inhibit proteolytic degradation during  
35 purification, and antibiotics may be included to prevent the growth of adventitious contaminants. One skilled in the art will appreciate that purification methods suitable for native sequence Apo-2 may require modification to account for changes in the character of Apo-2 or its variants upon expression in recombinant cell culture.

40                   7.   Covalent Modifications of Apo-2 Polypeptides

Covalent modifications of Apo-2 are included within the scope of this invention. One type of covalent modification of the Apo-2 is

5 introduced into the molecule by reacting targeted amino acid residues of the Apo-2 with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C- terminal residues of the Apo-2.

10 Derivatization with bifunctional agents is useful for crosslinking Apo-2 to a water-insoluble support matrix or surface for use in the method for purifying anti-Apo-2 antibodies, and vice-versa. Derivatization with one or more bifunctional agents will also be useful for crosslinking Apo-2 molecules to generate Apo-2 dimers. Such dimers may increase binding avidity and extend half-life of the molecule in vivo. Commonly used crosslinking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis-(succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)dithio]propioimide yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Patent Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

20 Other modifications include deamidation of glutamyl and asparaginyl residues to the corresponding glutamyl and aspartyl residues, respectively, hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the  $\alpha$ -amino groups of lysine, arginine, and histidine side chains [T.E. Creighton, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)], acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group. The modified forms of the residues fall within the scope of the present invention.

35 Another type of covalent modification of the Apo-2 polypeptide included within the scope of this invention comprises altering the native glycosylation pattern of the polypeptide. "Altering the native glycosylation pattern" is intended for purposes herein to mean deleting one or more carbohydrate moieties found in native sequence

40

5 Apo-2, and/or adding one or more glycosylation sites that are not present in the native sequence Apo-2.

10 Glycosylation of polypeptides is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-  
15 acetylglactosamine, galactose, or xylose to a hydroxylamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

20 Addition of glycosylation sites to the Apo-2 polypeptide may be accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the native sequence Apo-2 (for O-linked glycosylation sites). The Apo-2 amino acid sequence may optionally be altered through  
25 changes at the DNA level, particularly by mutating the DNA encoding the Apo-2 polypeptide at preselected bases such that codons are generated that will translate into the desired amino acids. The DNA mutation(s) may be made using methods described above and in U.S. Pat. No. 5,364,934, supra.  
30

Another means of increasing the number of carbohydrate moieties on the Apo-2 polypeptide is by chemical or enzymatic coupling of glycosides to the polypeptide. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free sulfhydryl groups such as those of cysteine,  
35 (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. These methods are described in WO 87/05330 published 11 September 1987, and in  
40 Aplin and Wriston, CRC Crit. Rev. Biochem., pp. 259-306 (1981).

Removal of carbohydrate moieties present on the Apo-2 polypeptide may be accomplished chemically or enzymatically or by

5 mutational substitution of codons encoding for amino acid residues that  
serve as targets for glycosylation. For instance, chemical  
deglycosylation by exposing the polypeptide to the compound  
trifluoromethanesulfonic acid, or an equivalent compound can result in  
10 the cleavage of most or all sugars except the linking sugar (N-  
acetylglucosamine or N-acetylgalactosamine), while leaving the  
polypeptide intact. Chemical deglycosylation is described by  
Hakimuddin, et al., Arch. Biochem. Biophys., 259:52 (1987) and by Edge  
et al., Anal. Biochem., 118:131 (1981). Enzymatic cleavage of  
15 carbohydrate moieties on polypeptides can be achieved by the use of a  
variety of endo- and exo-glycosidases as described by Thotakura et al.,  
Meth. Enzymol., 138:350 (1987).

Glycosylation at potential glycosylation sites may be  
prevented by the use of the compound tunicamycin as described by Duskin  
et al., J. Biol. Chem., 257:3105 (1982). Tunicamycin blocks the  
20 formation of protein-N-glycoside linkages.

Another type of covalent modification of Apo-2 comprises  
linking the Apo-2 polypeptide to one of a variety of nonproteinaceous  
polymers, e.g., polyethylene glycol, polypropylene glycol, or  
polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835;  
25 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

#### 8. Apo-2 Chimeras

The present invention also provides chimeric molecules  
comprising Apo-2 fused to another, heterologous polypeptide or amino  
acid sequence.

30 In one embodiment, the chimeric molecule comprises a fusion  
of the Apo-2 with a tag polypeptide which provides an epitope to which  
an anti-tag antibody can selectively bind. The epitope tag is generally  
placed at the amino- or carboxyl- terminus of the Apo-2. The presence  
of such epitope-tagged forms of the Apo-2 can be detected using an  
35 antibody against the tag polypeptide. Also, provision of the epitope  
tag enables the Apo-2 to be readily purified by affinity purification  
using an anti-tag antibody or another type of affinity matrix that binds  
to the epitope tag.

Various tag polypeptides and their respective antibodies are  
40 well known in the art. Examples include the flu HA tag polypeptide and  
its antibody 12CA5 [Field et al., Mol. Cell. Biol., 8:2159-2165 (1988)];  
the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto

5 [Evan et al., Molecular and Cellular Biology, 5:3610-3616 (1985)]; and  
the Herpes Simplex virus glycoprotein D (gD) tag and its antibody  
[Paborsky et al., Protein Engineering, 3(6):547-553 (1990)]. Other tag  
polypeptides include the Flag-peptide [Hopp et al., BioTechnology,  
6:1204-1210 (1988)]; the KT3 epitope peptide [Martin et al., Science,  
10 255:192-194 (1992)]; an  $\alpha$ -tubulin epitope peptide [Skinner et al., J.  
Biol. Chem., 266:15163-15166 (1991)]; and the T7 gene 10 protein peptide  
tag [Lutz-Freyermuth et al., Proc. Natl. Acad. Sci. USA, 87:6393-6397  
(1990)]. Once the tag polypeptide has been selected, an antibody  
thereto can be generated using the techniques disclosed herein.

15 Generally, epitope-tagged Apo-2 may be constructed and  
produced according to the methods described above. Epitope-tagged Apo-2  
is also described in the Examples below. Apo-2-tag polypeptide fusions  
are preferably constructed by fusing the cDNA sequence encoding the Apo-  
2 portion in-frame to the tag polypeptide DNA sequence and expressing  
20 the resultant DNA fusion construct in appropriate host cells.  
Ordinarily, when preparing the Apo-2-tag polypeptide chimeras of the  
present invention, nucleic acid encoding the Apo-2 will be fused at its  
3' end to nucleic acid encoding the N-terminus of the tag polypeptide,  
however 5' fusions are also possible. For example, a polyhistidine  
25 sequence of about 5 to about 10 histidine residues may be fused at the  
N- terminus or the C- terminus and used as a purification handle in  
affinity chromatography.

Epitope-tagged Apo-2 can be purified by affinity  
chromatography using the anti-tag antibody. The matrix to which the  
30 affinity antibody is attached may include, for instance, agarose,  
controlled pore glass or poly(styrenedivinyl)benzene. The epitope-  
tagged Apo-2 can then be eluted from the affinity column using  
techniques known in the art.

In another embodiment, the chimeric molecule comprises an  
35 Apo-2 polypeptide fused to an immunoglobulin sequence. The chimeric  
molecule may also comprise a particular domain sequence of Apo-2, such  
as the extracellular domain sequence of native Apo-2 fused to an  
immunoglobulin sequence. This includes chimeras in monomeric, homo- or  
heteromultimeric, and particularly homo- or heterodimeric, or -  
40 tetrameric forms; optionally, the chimeras may be in dimeric forms or  
homodimeric heavy chain forms. Generally, these assembled

- 5 immunoglobulins will have known unit structures as represented by the following diagrams.

5007419-020995

5

X or A  
 $\backslash$  \_\_\_\_\_ C<sub>H</sub> or C<sub>L</sub>

10

X or A  
 $\backslash$  \_\_\_\_\_ Y \_\_\_\_\_ C<sub>H</sub> or C<sub>L</sub>

15

A  
 A  $\backslash$  \_\_\_\_\_ C<sub>L</sub>  
 $\backslash$  \_\_\_\_\_ C<sub>H</sub>

20

A  
 V<sub>H</sub>  $\backslash$  \_\_\_\_\_ C<sub>L</sub>  
 $\backslash$  \_\_\_\_\_ C<sub>H</sub>

25

V<sub>L</sub>  
 A  $\backslash$  \_\_\_\_\_ C<sub>L</sub>  
 $\backslash$  \_\_\_\_\_ C<sub>H</sub>

30

X  
 A  $\backslash$  \_\_\_\_\_ C<sub>L</sub>  
 $\backslash$  \_\_\_\_\_ C<sub>H</sub>

35

A  
 X  $\backslash$  \_\_\_\_\_ C<sub>L</sub>  
 $\backslash$  \_\_\_\_\_ C<sub>H</sub>

A basic four chain structural unit is the form in which IgG, IgD, and IgE exist. A four chain unit is repeated in the higher molecular weight immunoglobulins; IgM generally exists as a pentamer of basic four-chain units held together by disulfide bonds. IgA globulin, and occasionally IgG globulin, may also exist in a multimeric form in

5 serum. In the case of multimers, each four chain unit may be the same or different.

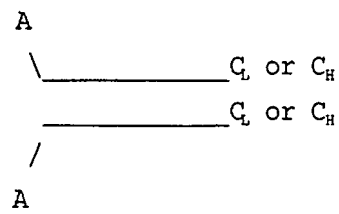
The following diagrams depict some exemplary monomer, homo- and heterodimer and homo- and heteromultimer structures. These diagrams are merely illustrative, and the chains of the multimers are believed to  
10 be disulfide bonded in the same fashion as native immunoglobulins.



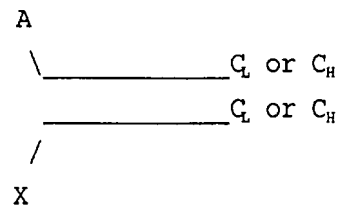
monomer:



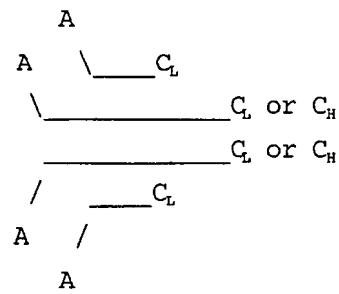
homodimer:



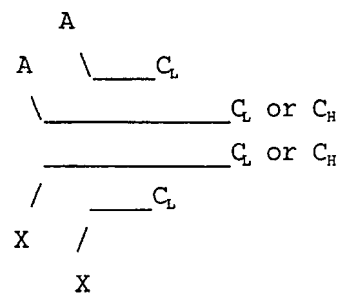
heterodimer:



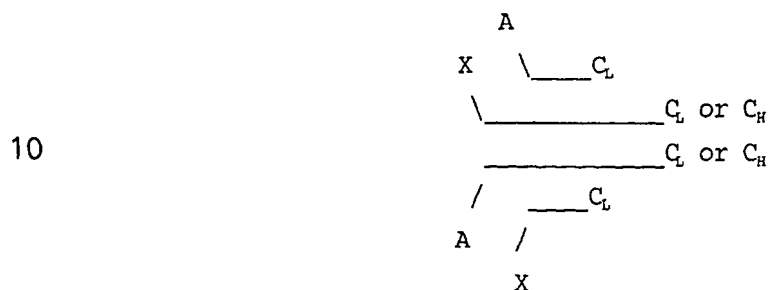
homotetramer:



heterotetramer:



5 and



In the foregoing diagrams, "A" means an Apo-2 sequence or an Apo-2 sequence fused to a heterologous sequence; X is an additional agent, which may be the same as A or different, a portion of an immunoglobulin superfamily member such as a variable region or a variable region-like domain, including a native or chimeric immunoglobulin variable region, a toxin such a pseudomonas exotoxin or ricin, or a sequence functionally binding to another protein, such as other cytokines (i.e., IL-1, interferon- $\gamma$ ) or cell surface molecules (i.e., NGFR, CD40, OX40, Fas antigen, T2 proteins of Shope and myxoma poxviruses), or a polypeptide therapeutic agent not otherwise normally associated with a constant domain; Y is a linker or another receptor sequence; and  $V_L$ ,  $V_H$ ,  $C_L$  and  $C_H$  represent light or heavy chain variable or constant domains of an immunoglobulin. Structures comprising at least one CRD of an Apo-2 sequence as "A" and another cell-surface protein having a repetitive pattern of CRDs (such as TNFR) as "X" are specifically included.

It will be understood that the above diagrams are merely exemplary of the possible structures of the chimeras of the present invention, and do not encompass all possibilities. For example, there might desirably be several different "A"s, "X"s, or "Y"s in any of these constructs. Also, the heavy or light chain constant domains may be originated from the same or different immunoglobulins. All possible permutations of the illustrated and similar structures are all within the scope of the invention herein.

In general, the chimeric molecules can be constructed in a fashion similar to chimeric antibodies in which a variable domain from

5 an antibody of one species is substituted for the variable domain of another species. See, for example, EP 0 125 023; EP 173,494; Munro, Nature, 312:597 (13 December 1984); Neuberger et al., Nature, 312:604-608 (13 December 1984); Sharon et al., Nature, 309:364-367 (24 May 1984); Morrison et al., Proc. Nat'l. Acad. Sci. USA, 81:6851-6855  
10 (1984); Morrison et al., Science, 229:1202-1207 (1985); Boulianne et al., Nature, 312:643-646 (13 December 1984); Capon et al., Nature, 337:525-531 (1989); Traunecker et al., Nature, 339:68-70 (1989).

Alternatively, the chimeric molecules may be constructed as follows. The DNA including a region encoding the desired sequence, such  
15 as an Apo-2 and/or TNFR sequence, is cleaved by a restriction enzyme at or proximal to the 3' end of the DNA encoding the immunoglobulin-like domain(s) and at a point at or near the DNA encoding the N-terminal end of the Apo-2 or TNFR polypeptide (where use of a different leader is contemplated) or at or proximal to the N-terminal coding region for TNFR  
20 (where the native signal is employed). This DNA fragment then is readily inserted proximal to DNA encoding an immunoglobulin light or heavy chain constant region and, if necessary, the resulting construct tailored by deletional mutagenesis. Preferably, the Ig is a human immunoglobulin when the chimeric molecule is intended for in vivo  
25 therapy for humans. DNA encoding immunoglobulin light or heavy chain constant regions is known or readily available from cDNA libraries or is synthesized. See for example, Adams et al., Biochemistry, 19:2711-2719 (1980); Gough et al., Biochemistry, 19:2702-2710 (1980); Dolby et al., Proc. Natl. Acad. Sci. USA, 77:6027-6031 (1980); Rice et al., Proc.  
30 Natl. Acad. Sci., 79:7862-7865 (1982); Falkner et al., Nature, 298:286-288 (1982); and Morrison et al., Ann. Rev. Immunol., 2:239-256 (1984).

Further details of how to prepare such fusions are found in publications concerning the preparation of immunoadhesins. Immunoadhesins in general, and CD4-Ig fusion molecules specifically are  
35 disclosed in WO 89/02922, published 6 April 1989). Molecules comprising the extracellular portion of CD4, the receptor for human immunodeficiency virus (HIV), linked to IgG heavy chain constant region are known in the art and have been found to have a markedly longer half-life and lower clearance than the soluble extracellular portion of CD4  
40 [Capon et al., supra; Byrn et al., Nature, 344:667 (1990)]. The construction of specific chimeric TNFR-IgG molecules is also described in Ashkenazi et al. Proc. Natl. Acad. Sci., 88:10535-10539 (1991);

- 5 Lesslauer et al. [J. Cell. Biochem. Supplement 15F, 1991, p. 115 (P 432)]; and Peppel and Beutler, J. Cell. Biochem. Supplement 15F, 1991, p. 118 (P 439)].

B. Therapeutic and Non-therapeutic Uses for Apo-2

10 Apo-2, as disclosed in the present specification, can be employed therapeutically to induce apoptosis in mammalian cells. This therapy can be accomplished for instance, using *in vivo* or *ex vivo* gene therapy techniques and includes the use of the death domain sequences disclosed herein. The Apo-2 chimeric molecules (including the chimeric  
15 molecules containing an extracellular domain sequence of Apo-2) comprising immunoglobulin sequences can also be employed therapeutically to inhibit apoptosis or NF-KB induction by Apo-2L or by another ligand that Apo-2 binds to.

20 The Apo-2 of the invention also has utility in non-therapeutic applications. Nucleic acid sequences encoding the Apo-2 may be used as a diagnostic for tissue-specific typing. For example, procedures like *in situ* hybridization, Northern and Southern blotting, and PCR analysis may be used to determine whether DNA and/or RNA encoding Apo-2 is present in the cell type(s) being evaluated. Apo-2  
25 nucleic acid will also be useful for the preparation of Apo-2 by the recombinant techniques described herein.

30 The isolated Apo-2 may be used in quantitative diagnostic assays as a control against which samples containing unknown quantities of Apo-2 may be prepared. Apo-2 preparations are also useful in generating antibodies, as standards in assays for Apo-2 (e.g., by labeling Apo-2 for use as a standard in a radioimmunoassay, radioreceptor assay, or enzyme-linked immunoassay), in affinity purification techniques, and in competitive-type receptor binding assays when labeled with, for instance, radioiodine, enzymes, or fluorophores.

35 Modified forms of the Apo-2, such as the Apo-2-IgG chimeric molecules (immunoadhesins) described above, can be used as immunogens in producing anti-Apo-2 antibodies.

40 Nucleic acids which encode Apo-2 or its modified forms can also be used to generate either transgenic animals or "knock out" animals which, in turn, are useful in the development and screening of therapeutically useful reagents. A transgenic animal (e.g., a mouse or

rat) is an animal having cells that contain a transgene, which transgene was introduced into the animal or an ancestor of the animal at a prenatal, e.g., an embryonic stage. A transgene is a DNA which is integrated into the genome of a cell from which a transgenic animal develops. In one embodiment, cDNA encoding Apo-2 or an appropriate sequence thereof (such as Apo-2-IgG) can be used to clone genomic DNA encoding Apo-2 in accordance with established techniques and the genomic sequences used to generate transgenic animals that contain cells which express DNA encoding Apo-2. Methods for generating transgenic animals, particularly animals such as mice or rats, have become conventional in the art and are described, for example, in U.S. Patent Nos. 4,736,866 and 4,870,009. Typically, particular cells would be targeted for Apo-2 transgene incorporation with tissue-specific enhancers. Transgenic animals that include a copy of a transgene encoding Apo-2 introduced into the germ line of the animal at an embryonic stage can be used to examine the effect of increased expression of DNA encoding Apo-2. Such animals can be used as tester animals for reagents thought to confer protection from, for example, pathological conditions associated with excessive apoptosis. In accordance with this facet of the invention, an animal is treated with the reagent and a reduced incidence of the pathological condition, compared to untreated animals bearing the transgene, would indicate a potential therapeutic intervention for the pathological condition. In another embodiment, transgenic animals that carry a soluble form of Apo-2 such as an Apo-2 ECD or an immunoglobulin chimera of such form could be constructed to test the effect of chronic neutralization of Apo-2L, a ligand of Apo-2.

Alternatively, non-human homologues of Apo-2 can be used to construct an Apo-2 "knock out" animal which has a defective or altered gene encoding Apo-2 as a result of homologous recombination between the endogenous gene encoding Apo-2 and altered genomic DNA encoding Apo-2 introduced into an embryonic cell of the animal. For example, cDNA encoding Apo-2 can be used to clone genomic DNA encoding Apo-2 in accordance with established techniques. A portion of the genomic DNA encoding Apo-2 can be deleted or replaced with another gene, such as a gene encoding a selectable marker which can be used to monitor integration. Typically, several kilobases of unaltered flanking DNA (both at the 5' and 3' ends) are included in the vector [see e.g., Thomas and Capecchi, Cell, 51:503 (1987) for a description of homologous

recombination vectors]. The vector is introduced into an embryonic stem cell line (e.g., by electroporation) and cells in which the introduced DNA has homologously recombined with the endogenous DNA are selected [see e.g., Li et al., Cell, 69:915 (1992)]. The selected cells are then injected into a blastocyst of an animal (e.g., a mouse or rat) to form aggregation chimeras [see e.g., Bradley, in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E. J. Robertson, ed. (IRL, Oxford, 1987), pp. 113-152]. A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term to create a "knock out" animal. Progeny harboring the homologously recombined DNA in their germ cells can be identified by standard techniques and used to breed animals in which all cells of the animal contain the homologously recombined DNA. Knockout animals can be characterized for instance, for their ability to defend against certain pathological conditions and for their development of pathological conditions due to absence of the Apo-2 polypeptide, including for example, development of tumors.

### C. Anti-Apo-2 Antibody Preparation

The present invention further provides anti-Apo-2 antibodies.

Antibodies against Ap0-2 may be prepared as follows. Exemplary antibodies include polyclonal, monoclonal, humanized, bispecific, and heteroconjugate antibodies.

## 1. Polyclonal Antibodies

The Apo-2 antibodies may comprise polyclonal antibodies.

Methods of preparing polyclonal antibodies are known to the skilled artisan. Polyclonal antibodies can be raised in a mammal, for example, by one or more injections of an immunizing agent and, if desired, an adjuvant. Typically, the immunizing agent and/or adjuvant will be injected in the mammal by multiple subcutaneous or intraperitoneal injections. The immunizing agent may include the Apo-2 polypeptide or a fusion protein thereof. An example of a suitable immunizing agent is an Apo-2-IgG fusion protein, such as an Apo-2 ECD-IgG fusion protein. Cells expressing Apo-2 at their surface may also be employed. It may be useful to conjugate the immunizing agent to a protein known to be immunogenic in the mammal being immunized. Examples of such immunogenic proteins which may be employed include but are not limited to keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, and soybean

5 trypsin inhibitor. An aggregating agent such as alum may also be  
employed to enhance the mammal's immune response. Examples of adjuvants  
which may be employed include Freund's complete adjuvant and MPL-TDM  
adjuvant (monophosphoryl Lipid A, synthetic trehalose dicorynomycolate).  
10 The immunization protocol may be selected by one skilled in the art  
without undue experimentation. The mammal can then be bled, and the  
serum assayed for antibody titer. If desired, the mammal can be boosted  
until the antibody titer increases or plateaus.

## 2. Monoclonal Antibodies

15 The Apo-2 antibodies may, alternatively, be monoclonal  
antibodies. Monoclonal antibodies may be prepared using hybridoma  
methods, such as those described by Kohler and Milstein, supra. In a  
hybridoma method, a mouse, hamster, or other appropriate host animal, is  
typically immunized (such as described above) with an immunizing agent  
to elicit lymphocytes that produce or are capable of producing  
20 antibodies that will specifically bind to the immunizing agent.  
Alternatively, the lymphocytes may be immunized in vitro.

30 The immunizing agent will typically include the Apo-2  
polypeptide or a fusion protein thereof. An example of a suitable  
immunizing agent is an Apo-2-IgG fusion protein or chimeric molecule. A  
specific example of an Apo-2 ECD-IgG immunogen is described in Example 9  
below. Cells expressing Apo-2 at their surface may also be employed.  
Generally, either peripheral blood lymphocytes ("PBLs") are used if  
cells of human origin are desired, or spleen cells or lymph node cells  
are used if non-human mammalian sources are desired. The lymphocytes  
35 are then fused with an immortalized cell line using a suitable fusing  
agent, such as polyethylene glycol, to form a hybridoma cell [Goding,  
Monoclonal Antibodies: Principles and Practice, Academic Press, (1986)  
pp. 59-103]. Immortalized cell lines are usually transformed mammalian  
cells, particularly myeloma cells of rodent, bovine and human origin.  
40 Usually, rat or mouse myeloma cell lines are employed. The hybridoma  
cells may be cultured in a suitable culture medium that preferably  
contains one or more substances that inhibit the growth or survival of  
the unfused, immortalized cells. For example, if the parental  
transformed cells lack the enzyme hypoxanthine guanine phosphoribosyl  
transferase (HGPRT or HPRT), the culture medium for the hybridomas  
typically will include hypoxanthine, aminopterin, and thymidine ("HAT  
medium"), which substances prevent the growth of HGPRT-deficient cells.

5 Preferred immortalized cell lines are those that fuse efficiently, support stable high level expression of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. More preferred immortalized cell lines are murine myeloma lines, which can be obtained, for instance, from the Salk Institute Cell  
10 Distribution Center, San Diego, California and the American Type Culture Collection, Rockville, Maryland. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies [Kozbor, J. Immunol., 133:3001 (1984); Brodeur et al., Monoclonal Antibody Production Techniques and  
15 Applications, Marcel Dekker, Inc., New York, (1987) pp. 51-63].

The culture medium in which the hybridoma cells are cultured can then be assayed for the presence of monoclonal antibodies directed against Apo-2. Preferably, the binding specificity of monoclonal antibodies produced by the hybridoma cells is determined by  
20 immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA). Such techniques and assays are known in the art. The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson and Pollard, Anal. Biochem., 107:220  
25 (1980).

After the desired hybridoma cells are identified, the clones may be subcloned by limiting dilution procedures and grown by standard methods [Goding, supra]. Suitable culture media for this purpose  
30 include, for example, Dulbecco's Modified Eagle's Medium and RPMI-1640 medium. Alternatively, the hybridoma cells may be grown in vivo as ascites in a mammal.

The monoclonal antibodies secreted by the subclones may be isolated or purified from the culture medium or ascites fluid by conventional immunoglobulin purification procedures such as, for  
35 example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

The monoclonal antibodies may also be made by recombinant DNA methods, such as those described in U.S. Patent No. 4,816,567. DNA encoding the monoclonal antibodies of the invention can be readily  
40 isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The



5 hybridoma cells of the invention serve as a preferred source of such  
DNA. Once isolated, the DNA may be placed into expression vectors,  
which are then transfected into host cells such as simian COS cells,  
Chinese hamster ovary (CHO) cells, or myeloma cells that do not  
10 otherwise produce immunoglobulin protein, to obtain the synthesis of  
monoclonal antibodies in the recombinant host cells. The DNA also may  
be modified, for example, by substituting the coding sequence for human  
heavy and light chain constant domains in place of the homologous murine  
sequences [U.S. Patent No. 4,816,567; Morrison et al., supra] or by  
15 covalently joining to the immunoglobulin coding sequence all or part of  
the coding sequence for a non-immunoglobulin polypeptide. Such a non-  
immunoglobulin polypeptide can be substituted for the constant domains  
of an antibody of the invention, or can be substituted for the variable  
domains of one antigen-combining site of an antibody of the invention to  
create a chimeric bivalent antibody.

20 As described in the Examples below, anti-Apo-2 monoclonal  
antibodies have been prepared. One of these antibodies, 3F11.39.7, has  
been deposited with ATCC and has been assigned deposit accession no. HB-  
12456. In one embodiment, the monoclonal antibodies of the invention  
will have the same biological characteristics as the monoclonal  
25 antibodies secreted by the hybridoma cell line(s) deposited under  
Accession No. HB-12456. The term "biological characteristics" is used  
to refer to the *in vitro* and/or *in vivo* activities or properties of the  
monoclonal antibody, such as the ability to specifically bind to Apo-2  
or to substantially block, induce or enhance Apo-2 activation. As  
30 disclosed in the present specification, the 3F11.39.7 monoclonal  
antibody (HB-12456) is characterized as having agonistic activity for  
inducing apoptosis, binding to the Apo-2 receptor, having blocking  
activity as described in the Examples below, and having some cross-  
reactivity to DR4 but not to DcR1 or DcR2. Optionally, the monoclonal  
35 antibody will bind to the same epitope as the 3F11.39.7 antibody  
disclosed herein. This can be determined by conducting various assays,  
such as described herein and in the Examples. For instance, to  
determine whether a monoclonal antibody has the same specificity as the  
3F11.39.7 antibody specifically disclosed, one can compare activity in  
40 Apo-2 blocking and apoptosis induction assays, such as those described  
in the Examples below.

The antibodies of the invention may also comprise monovalent

5 antibodies. Methods for preparing monovalent antibodies are well known  
in the art. For example, one method involves recombinant expression of  
immunoglobulin light chain and modified heavy chain. The heavy chain is  
truncated generally at any point in the Fc region so as to prevent heavy  
chain crosslinking. Alternatively, the relevant cysteine residues are  
10 substituted with another amino acid residue or are deleted so as to  
prevent crosslinking.

In vitro methods are also suitable for preparing monovalent  
antibodies. Digestion of antibodies to produce fragments thereof,  
particularly, Fab fragments, can be accomplished using routine  
15 techniques known in the art. For instance, digestion can be performed  
using papain. Examples of papain digestion are described in WO 94/29348  
published 12/22/94 and U.S. Patent No. 4,342,566. Papain digestion of  
antibodies typically produces two identical antigen binding fragments,  
called Fab fragments, each with a single antigen binding site, and a  
20 residual Fc fragment. Pepsin treatment yields an F(ab')<sub>2</sub> fragment that  
has two antigen combining sites and is still capable of cross-linking  
antigen.

The Fab fragments produced in the antibody digestion also  
contain the constant domains of the light chain and the first constant  
25 domain (CH<sub>1</sub>) of the heavy chain. Fab' fragments differ from Fab  
fragments by the addition of a few residues at the carboxy terminus of  
the heavy chain CH<sub>1</sub> domain including one or more cysteines from the  
antibody hinge region. Fab'-SH is the designation herein for Fab' in  
which the cysteine residue(s) of the constant domains bear a free thiol  
30 group. F(ab')<sub>2</sub> antibody fragments originally were produced as pairs of  
Fab' fragments which have hinge cysteines between them. Other chemical  
couplings of antibody fragments are also known.

### 3. Humanized Antibodies

The Apo-2 antibodies of the invention may further comprise  
35 humanized antibodies or human antibodies. Humanized forms of non-human  
(e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin  
chains or fragments thereof (such as Fv, Fab, Fab', F(ab')<sub>2</sub> or other  
antigen-binding subsequences of antibodies) which contain minimal  
sequence derived from non-human immunoglobulin. Humanized antibodies  
40 include human immunoglobulins (recipient antibody) in which residues  
from a complementary determining region (CDR) of the recipient are  
replaced by residues from a CDR of a non-human species (donor antibody)

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5 such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin [Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature, 332:323-329 (1988); and Presta, Curr. Op. Struct. Biol., 2:593-596 (1992)].

20 Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers [Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature, 332:323-327 (1988); Verhoeyen et al., Science, 239:1534-1536 (1988)], by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Patent No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

35 The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important in order to reduce antigenicity. According to the "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody [Sims et al., J.

5 Immunol., 151:2296 (1993); Chothia and Lesk, J. Mol. Biol., 196:901  
(1987)]. Another method uses a particular framework derived from the  
consensus sequence of all human antibodies of a particular subgroup of  
light or heavy chains. The same framework may be used for several  
different humanized antibodies [Carter et al., Proc. Natl. Acad. Sci.  
10 USA, 89:4285 (1992); Presta et al., J. Immunol., 151:2623 (1993)].

It is further important that antibodies be humanized with  
retention of high affinity for the antigen and other favorable  
biological properties. To achieve this goal, according to a preferred  
method, humanized antibodies are prepared by a process of analysis of  
15 the parental sequences and various conceptual humanized products using  
three dimensional models of the parental and humanized sequences. Three  
dimensional immunoglobulin models are commonly available and are  
familiar to those skilled in the art. Computer programs are available  
which illustrate and display probable three-dimensional conformational  
20 structures of selected candidate immunoglobulin sequences. Inspection  
of these displays permits analysis of the likely role of the residues in  
the functioning of the candidate immunoglobulin sequence, i.e., the  
analysis of residues that influence the ability of the candidate  
immunoglobulin to bind its antigen. In this way, FR residues can be  
25 selected and combined from the consensus and import sequence so that the  
desired antibody characteristic, such as increased affinity for the  
target antigen(s), is achieved. In general, the CDR residues are  
directly and most substantially involved in influencing antigen binding  
[see, WO 94/04679 published 3 March 1994].

30 Transgenic animals (e.g., mice) that are capable, upon  
immunization, of producing a full repertoire of human antibodies in the  
absence of endogenous immunoglobulin production can be employed.  
Transfer of the human germ-line immunoglobulin gene array in such germ-  
line mutant mice will result in the production of human antibodies upon  
35 antigen challenge [see, e.g., Jakobovits et al., Proc. Natl. Acad. Sci.  
USA, 90:2551-255 (1993); Jakobovits et al., Nature, 362:255-258 (1993);  
Bruggemann et al., Year in Immuno., 7:33 (1993)]. Human antibodies can  
also be produced in phage display libraries [Hoogenboom and Winter, J.  
Mol. Biol., 227:381 (1991); Marks et al., J. Mol. Biol., 222:581  
40 (1991)]. The techniques of Cole et al. and Boerner et al. are also  
available for the preparation of human monoclonal antibodies (Cole et  
al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77

5 (1985) and Boerner et al., J. Immunol., 147(1):86-95 (1991)].

#### 4. Bispecific Antibodies

Bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens. In the present case, one of the binding  
10 specificities is for the Apo-2, the other one is for any other antigen, and preferably for a cell-surface protein or receptor or receptor subunit.

Methods for making bispecific antibodies are known in the art. Traditionally, the recombinant production of bispecific antibodies  
15 is based on the co-expression of two immunoglobulin heavy-chain/light-chain pairs, where the two heavy chains have different specificities [Milstein and Cuello, Nature, 305:537-539 (1983)]. Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of ten different  
20 antibody molecules, of which only one has the correct bispecific structure. The purification of the correct molecule is usually accomplished by affinity chromatography steps. Similar procedures are disclosed in WO 93/08829, published 13 May 1993, and in Traunecker et al., EMBO J., 10:3655-3659 (1991).

According to a different and more preferred approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy-chain constant domain, comprising at least part of the hinge, CH2,  
30 and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light-chain binding present in at least one of the fusions. DNAs encoding the immunoglobulin heavy-chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-  
35 transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three  
40 polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance. In a preferred

5 embodiment of this approach, the bispecific antibodies are composed of a  
hybrid immunoglobulin heavy chain with a first binding specificity in  
one arm, and a hybrid immunoglobulin heavy-chain/light-chain pair  
(providing a second binding specificity) in the other arm. It was found  
10 that this asymmetric structure facilitates the separation of the desired  
bispecific compound from unwanted immunoglobulin chain combinations, as  
the presence of an immunoglobulin light chain in only one half of the  
bispecific molecule provides for a facile way of separation. This  
approach is disclosed in WO 94/04690 published 3 March 1994. For further  
15 details of generating bispecific antibodies see, for example, Suresh et  
al., Methods in Enzymology, 121:210 (1986).

#### 5. Heteroconjugate Antibodies

Heteroconjugate antibodies are also within the scope of the  
present invention. Heteroconjugate antibodies are composed of two  
covalently joined antibodies. Such antibodies have, for example, been  
20 proposed to target immune system cells to unwanted cells [US Patent No.  
4,676,980], and for treatment of HIV infection [WO 91/00360; WO  
92/200373; EP 03089]. It is contemplated that the antibodies may be  
prepared in vitro using known methods in synthetic protein chemistry,  
including those involving crosslinking agents. For example,  
25 immunotoxins may be constructed using a disulfide exchange reaction or  
by forming a thioether bond. Examples of suitable reagents for this  
purpose include iminothiolate and methyl-4-mercaptobutyrimidate and  
those disclosed, for example, in U.S. Pat. No. 4,676,980.

#### 6. Triabodies

30 Triabodies are also within the scope of the invention. Such  
antibodies are described for instance in Iliades et al., FEBS Letters,  
409:437-441 (1997) and Korrt et al., Protein Engineering, 10:423-433  
(1997).

#### 7. Other Modifications

35 Other modifications of the Apo-2 antibodies are contemplated.  
For example, it may be desirable to modify the antibodies of the  
invention with respect to effector function, so as to enhance the  
therapeutic effectiveness of the antibodies. For instance, cysteine  
residue(s) may be introduced into the Fc region, thereby allowing  
40 interchain disulfide bond formation in this region. The homodimeric  
antibody thus generated may have improved internalization capability  
and/or increased complement-mediated cell killing [see, e.g., Caron et

< /home/ruby/va/Molbio/sam/sequences/tnfngfrlike/deathdomain/new/ss.Con.27868 12-40  
 < Sequence of entire insert of clone 27868 (A.K.A., DD.P.2) -GT- AG  
 < Sequenced by Christa Gray and Sherry Heldons  
 < Requested by Scot Marsters and Avi Ashkenazi.  
 < length: 1827

1 GATCCCTCGA CCTCGACCCA CGCGTCCGCC CACGCGTCCG CATAAATCAG CACGCGCGCG GAGAACCCCG CAATCTCTGC GCCACAAAA TACACCGAGC  
 CTAGGGAGCT GGAGCTGGGT GCGCAGGCGG GTGCGCAGGC GTATTAGTC GTGCGCGCGC CTCTTGGGC GTTAGAGACG CCGGTGTTTT ATGTGGCTGC  
 1 AspProSer hrSerThrHi salaserAla HisAlaSerA laOC\*ileSe rThrArgPro GluAsnProA laileSerAl aProThrLys TyrThrAspAsp  
 IleProArg proArgProT hrArgProPr oThrArgPro HisLysSerA laArgGlyAr gArgThrPro GlnSerLeuA rgProGlnAs nThrProThr  
 SerLeuAs pLeuAspPro ArgValArgP roArgValAr gileAsnGln HisAlaAlaG lyGluProAr gAsnLeuCys AlaHisLysI leHisArgArg  
 101 ATGCCCCGATC TACTTTAAGG GCTGAACACC ACGGGCCTGA GAGACTATAA GAGCGTTCCC TACCGCCATG GAACAACGGG GACAGAACGC CCCGGCCGCT  
 TACGGGCTAG ATGAATTC CGACTTTGGG TGCCCGGACT CTCTGATATT CTGCAAGGG ATGGCGGTAC CTTGTTGCC CTGTCTTGG GGGCCGGCGA  
 35 AlaArgSe rThrLeuArg AlaGluThrH isGlyProG l uArgLeuOC\* GluArgSerL euProProT r pAsnAsnGly AspArgThrP roArgProLeu  
 MetProAspL euLeuOC\*G l yLeuLysPro ThrGlyLeuA rgAspTyrLy sSerValPro TyrArgHisG lyThrThrGl yThrGluArg ProGlyArgPhe  
 CysProile TyrPheLysG lyOP\*AsnPr oArgAlaOP\* GluThrileA rgAlaPhePr oThrAla GluGlnArgG lyGlnAsnAl aProAlaAla  
 201 TCGGGGGCCC GGAANAAGGA CGGCCACAGG ACGGGGGAGG CGCGGGGAGC CAGGCGTGGG CTCCGGGTCC CCAAGACCCCT TGTGCTCGTT GTCCGCGCGG  
 AGCCCCCGG CCTTTTCCT GCGGGTCTCT GCGTCCCTCC GCGCCCTCG GTCGGGACCC GAGGCCAGG GGTCTGGGA ACACGAGCAA CAGCGCGCGC  
 68 ArgGlyPro GlyLysGlyT hrAlaGlnAs pProGlyArg ArgGlyGluP roGlyLeuG l ySerGlySer ProArgProL euCysSerLe uSerProArg  
 GlyGlyPr oGluLysAla ArgProArgT hrGlnGlyG l yAlaGlySer GlnAlaTrpA laProGlyPr oGlnAspPro CysAlaArgC ysArgArgGly  
 SerGlyAlaA rgLysArgHi sGlyProGly ProArgGluA laArgGlyAl aArgProGly LeuArgValP roLysThrLe uValleuVal ValAlaAlaVal  
 301 TCCTGCTGT GGTCTAGCT GAGTCTGCTC ACCATATCTC AGAAGACGGT AGAGATTGCA TCCTCTGCAA ATATGGACAG GACTATAGCA CTCACCTGAA TGACCTCCTT  
 AGGACGACAA CCAGAGTCGA CTCAGACGAG ACTAGTGGGT TGTTCTGGAT CGAGGGTGC TCTCTGCGG GGTGTGTGTT TTCTCCAGGT CCGGGAGTCT  
 101 SerCysCyst rpSerGlnLe uSerLeuLeu OP\*SerProA snLysThrAM \*LeuProSer ArgGluArgP roHisAsnLy sArgGlyPro AlaProGlnArg  
 ProAlaVal GlyLeuSero p\*valCysSe rAspHisPro ThrArgProS erSerProAl aGluSerGly ProThrThrL ysGluValG l nProLeuArg  
 LeuLeuLe uValSerAla GluSerAlaL euilleThrG l nGlnAspLeu AlaProGlnG lnArgAlaAl aProGlnGln LysArgSerS erProSerGlu  
 401 GGGATTGTG CCACCTGGAC ACCATATCTC AGAAGACGGT AGAGATTGCA TCCTCTGCAA ATATGGACAG GACTATAGCA CTCACCTGAA TGACCTCCTT  
 CCTAACACA GGTGGACCTG TGGTATAGAG TCTTCTGCCA TCTCTAACGT AGAGGACGTT TATACCTGTC CTGATATCCT GAGTGACCTT ACTGGAGGAA  
 135 AspCysVa lHisLeuAsp ThrileSerG l nLysThrVa lGluileAla SerPrbAlaA snMetAspAr gThrileAla LeuThrGlyM etThrSerPhe  
 GlyileValS erThrTrpTh rProTyrLeu ArgArgArgA M\*ArgLeuHi sLeuLeuGln lletpThrG lyLeuAM\*Hi sSerLeuGlu OP\*ProProphe  
 GlyLeuCys ProProGlyH isHisileSe rGluAspGly ArgAspCysI leSerPysLy sTyrGlyGln AspTyrSert hrHisTrpAs nAspLeuLeu  
 501 TTCTGCTTG CTTGCCACAG GTGTGATTCA GGTGAAGTGG AGCTAAGTCC CTGACCCACG ACCAGAAACA CAGTGTGTCA GTCCGAGAA GGCACCTTCC  
 AAGACGACG CGACGTGGTC CACACTAAGT CCACCTTACC TCGATTACG GACGTGGTGC TGGTCTTGT GTACACACAGT CACGCTTCTT CCGTGGAAAG  
 168 SerAlaCys AlaAlaProG lyValileG l nVallyStrp SerOC\*ValP roAlaProAr gProGluThr GlnCysValS erAlaLysLy salaProSer  
 LeuLeuAl aleuHisGln ValOP\*Phea rgOP\*SerG l yAlaLysSer LeuHisHisA spGlnLysHi sSerValSer ValArgArgA rgHisLeuPro  
 PheCysLeuA rgCysThrAr gCysAspSer GlyGluValG luleuSerPr oCysThrThr ThrArgAsnT hrValCysG l nCysGluGlu GlyThrPheArg  
 601 GGAAGAAGA TTCTCTGAG ATGTGCCGA AGTGCCGCAC AGGGTGTCC AGAGGATGG TCAAGGTCCG TGATTGTACA CCCTGGAGTG ACATCGAATG  
 CCTTCTTCT AAGAGGACTC TACACGGCCT TCACGGCGTG TCCACAGGG TCTCCCTACC AGTCCAGCC ACTAACATGT GGGACCTCAC TGTAGCTTAC  
 201 GlyLysLysI leLeuLeuA rgCysAlaGly SerAlaAlaG l nGlyValPr oGluGlyTrp SerArgServ alileValHi sProGlyVal ThrSerAsnVal  
 GlyArgArg PheSerOP\*A spValProG l uValProHis ArgValserG l nArgAspG l yGlnGlyArg OP\*LeuTyrT hrLeuGluOP \*HisArgMet  
 GluGluAs pSerProGlu MetCysArgL ysCysArgTh rGlyCysPro ArgGlyMetV allysValG l yAspCysThr ProTrpSera spileGluCys

701 TGTCACAAA GAATCAGGCA TCATCATAGG AGTCACAGTT GCAGCCGTAG TCTTGATTGT GGCTGTGTTT GTTTGCAAGT CTTTACTGTG GAAGAAAAGTC  
 ACAGGTGTTT CTTAGTCCGT AGTAGTATCC TCAGTGTCAA CGTGGCATC AGAATAACA CCGACACAAA CAAACGTTCA GAAATGACAC CTTCTTTTACAG  
 235 SerThrLy sAsnGlnAla SerSerAM\*G luSerGlnLe uGlnProAM\* SerOP\*LeuT rpleuCysle uPheAlaser LeuTyrcysG lyArgLysSer  
 CysProGlnA rgileArgHi sHisHisArg SerHisSerC ysSerArgSe rLeuAspCys GlyCysValc ysleuGlnVa lPheThrVal GluGluSerPro  
 ValHisLys GluSerGlyI leilleileGI xValThrVal AlaAlaValV alleuilleva lAlaValPhe ValCysLysS erLeuLeuTr pLysLysVal

801 CTTCTTACC TGAAGGCAT CTGCTCAGGT GGTGGTGGG ACCTGAGCG TGTGGACAGA AGCTCACAAAC GACCTGGGGC TGAGGACAAT GTCCTCAATG  
 GAAGGAATGG ACTTTCCGTA GACGAGTCCA CCACCACCCC TGGGACTCGC ACACCTGTCT TCGAGTGTG CTGGACCCCG ACTCCTGTTA CAGGAGTTAC  
 268 PheLeuThr OP\*LysAlas erAlaGlnVa lValValGLY ThrLeuSerV alThrThrGL uAlaHisasn AspLeuGLY euArgThrMe tSerSerMet  
 SerLeuPr oGluArgHis LeuLeuArgT rPrTrpGL yProOP\*Ala CysGLYGLNl ysLeuThrTh rThrTrpGLy OP\*GlyGLNc ysProGLNOP\*  
 LeuProTyrl euLysGLYI eCysSerGLY GlyGLYGLY spProGluAr gValAspArg SerSerGlnA rgProGLYAl aGluAsn ValLeuAsnGlu

901 AGATCGTGAG TATCTTGCAG CCCACCCAGG TCCTTGAGCA GGAATGGAA GTCCAGGAGC CAGCAGAGCC AACAGGTGTC AACATGTTGT CCCCCGGGGA  
 TCTAGCACTC ATAGAACGTC GGTGGGTCC AGGACTCGT CCTTTACCTT CAGGTCTCG GTCGTCTCG TTGTCCACAG TTGTACAACA GGGGGCCCCCT  
 301 ArgSerOP\*v alSerCysse rProProArg SerLeuSerA rGLystrpLy sSerArgSer GlnGlnSerG lInGlnValSe rThrCysCys ProProGLYSer  
 AspArgGlu TyrLeuAlaa laHisProGL yProOP\*Ala GlyAsnGLYS erProGLYAl aSerArgAla AsnArgCysG lInHisValVa lProArgGLY  
 llevalse rleLeuGln ProThrGlnV alProGLuGL nGluMetGlu ValGlnGluP roAlaGluPr oThrGlyVal AsnMetLeuS erProGLYGLu

1001 GTCAGAGCAT CTGCTGGAAC CGGCAGAAAGC TGAAGGTCT CAGAGGAGGA GGCTGCTGCT TCCAGCAAAAT GAAGGTGATC CCACTGAGAC TCTGAGACAG  
 CAGTCTCGTA GACGACCTTG GCCGTCTTCG ACTTTCCAGA GTCTCTCTCT CCGACGACCA AGGTCTGTTA CTTCCACTAG GGTGACTCTG AGACTCTGTC  
 335 GlnSerII eCysTrpAsn ArgGlnLysL euLysGLYle uArgGLYGLY GlyCysTrpP heGlnGlnMe tLysValile ProLeuArgL euOP\*AspSer  
 ValArgAlas erAlaGLYTh rGlyArgSer OP\*LysValS erGluGluGL uAlaAlaGLY SerSerLySO P\*ArgOP\*Se rHisOP\*Asp SerGluThrVal  
 SerGluHis LeuLeuGluP roAlaGluAl aGluArgSer GlnArgArga rgLeuLeuVa lProAlaAsn GluGLYAspP roThrGluTh rLeuArgGln

1101 TGCTTCGATG ACTTTCGAGA CTTGGTGCCC TTTGACTCCT GGGAGCCGCT CATGAGGAAG TTGGGCCTCA TGGACAATGA GATAAAGGTG GCTAAAGCTG  
 ACGAAGCTAC TGAACGCTT TGAACGCTT GAAACGCTT GAACTGAGGA CCTCGGCGA GTACTCCTTC AACCCGGAGT ACCTGTTACT CTATTTCCAC CGATTTCGAC  
 368 AlaSerMet ThrLeuGlnT hrTrpCysPr oLeuThrPro GlySerArgS erOP\*GlySe rTrpAlaser TrpThrMeta rGOC\*ArgTr pleuLysLeu  
 LeuArgOP \*LeuCysArg leuGlyAlaL euOP\*LeuLe uOP\*LeuLe PheAspSerI rpGluProLe uMetArgLys LeuGLYLeum etAspAsnGL uileLysVal AlaLysAlaGlu  
 CysPheAspa sPheAlaAs pleuValPro PheAspSerI rpGluProLe uMetArgLys LeuGLYLeum etAspAsnGL uileLysVal AlaLysAlaGlu

1201 AGCAGCGGG CCACAGGGAC ACCTTGATCA CCAAGCAGAA GATTGAGGAC CACTTGTGTA GCTCTGAAA GTTCATGTAT CTAGAAGGTA ATGCAGACTC TGCCCTGTCC  
 TCCGTCGCC GGTGTCCTG TGAACATGT TGAACATGT GGTGTCCTG CTAACCTCTG GTGAACAACT CGAGACCTTT CAAGTACATA GATCTTCCAT TACGTCTGAG ACGGAACAGG  
 401 ArgGlnArgA laThrGlyTh rProCysThr ArgCysOP\*O C\*SerGlySe rThrLysPro GlyGluMetP roLeuSerTh rProCysTrp MetProTrpArg  
 GlySerGly ProGlnGlyH isLeuValHi sAspAlaAsp LysValGLY lInGlnAsnAr gAlaArgCys LeuCysProH isProAlaGL yCysLeuGLY  
 AlaAlaGL yHisArgasp ThrLeuTyrt hrMetLeuIl eLysTrpVal AsnLysThrG lyArgAspAl aSerValHis ThrLeuLeuA spAlaLeuGLu

1301 GACGCTGGGA GAGAGACTTG CCAAGCAGAA GATTGAGGAC CACTTGTGTA GCTCTGAAA GTTCATGTAT CTAGAAGGTA ATGCAGACTC TGCCCTGTCC  
 CTGCGACCCCT CTCTCTGAAC GGTTCGCTCTT CTAACCTCTG GTGAACAACT CGAGACCTTT CAAGTACATA GATCTTCCAT TACGTCTGAG ACGGAACAGG  
 435 ArgTrpGL uArgAspLeu ProSerArga rgLeuArgTh rThrCysOP\* AlaLeuGluS erSerCysIl eAM\*LysVal MetGlnThrL euProCysPro  
 AspAlaGLYa rgGluThrCy sGlnAlaGlu AspOP\*GLYp roLeuValGL lLeuTrpLys ValHisValS erArgArgOC \*CysArgLeu CysLeuValLeu  
 ThrLeuGLY GluArgLeuA laLysGlnLy sileGluAsp HisLeuLeuS erSerGlyLy sPheMetTytr LeuGluGLYa snAlaAspSe rAlaLeuSer

1401 TAAGTGTGAT TCTCTTCCAG AAGTGAGACC TTCCCTGTTT TACCTTTTTT CTGGAAAAAG CCCAACTGGA CTCCAGTCAG TAGGAAAAGTG CCACAATTGT  
 ATTACACTA AGAGAAGTCC TTCACCTCTG AAGGGACCAA ATGGAAAAA GACCTTTTTC GGGTTGACCT GAGGTCAGTC ATCCTTTTAC GGTGTTAACA  
 468 LysCysAsp SerLeuGlnG luValArgPr oSerLeuVal TyrLeuPheS erGLYLysSe rProThrGLY LeuGlnServ alGLYLysCy sHisAsnCys  
 SerValII eLeuPheArg LysOP\*AspL euProTrpPh eThrPhePhe LeuGluLysA laGlnLeuAs pSerSerGln AM\*GluSera laThrIleVal  
 OC\*ValOP\*P heserSerGI ySerGluThr PheProGLYL euProPhePh eTrpLysLys ProAsnTrpT hrProValse rArgLysVal ProGlnLeuSer

TM?

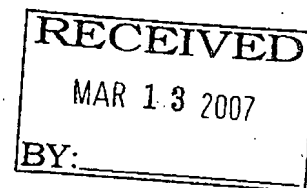
Death domain

1-283



1501 CACATGACCG GTACTGGAAG AAACCTCTCC ATCCAACATC ACCCAGTGGA TGGAACATCC TGTAACTTTT CACTGCACCT GGCATTATTT TTATAAGCTG  
GTGTACTGGC CATGACCTTC TTTGAGAGGG TAGGTTGTAG TGGGTCACCT ACCTTGTAGG ACATTGAAA CATTGACGTGAA CCGTAATAAA AATATTGAC  
501 HisMetThrG lyThrGlyAr gAsnSerPro ileGlnHisH isProValAs pGlyThrSer CysAsnPhes erLeuHisLe uAlaLeuPhe LeuOC\*AlaGlu  
ThrOP\*Pro ValLeuGluG luThrLeuPr oSerAsnile ThrGlnTrpM etGluHisPr oValThrPhe HisCysThrT rpHistyrPh etyrLysLeu  
HisAspAr gTyrTrpLys LysLeuSerH isProThrSe rProSerGly TrpAsnileL euOC\*LeuPh eThrAlaLeu GlyIleileP heIleSerOP\*  
1601 AATGTGATAA TAAGGACACT ATGGAATGT CTGGATCATT CCGTTTGTGC GTACTTTGAG ATTTGGTTTG GGATGTCATT GTTTTCACAG CACTTTTTTA  
TTACACTATT ATTCCTGTGA TACCTTTACA GACCTAGTAA GGCAACACACG CATGAAACTC TAACCACAAAC CCTACAGTAA CAAAAGTGTC GTGAAAAAAT  
535 CysAspAs nLysAspThr MetGluMets erGlySerPh eArgLeuCys ValLeuOP\*A sPLeuValTr pAspValile ValPheThrA laLeuPheTyr  
AsnValilei leArgThrLe uTrpLysCys LeuAspHisS erValCysAl aTyrPheGlu ileTrpPheG lyMetSerLe uPheSerGln HisPhePheIle  
MetOP\*OC\* OC\*GlyHist yrGlyAsnVa lTrpIleIle PropheValA rgThrLeuAr gPheGlyLeu GlyCysHisC ysPheHisSe rThrPheLeu  
1701 TCCTAATGTA AATGCTTTAT TTATTTATTT GGGCTACATT GTAAGATCCA TCTACAAAAA AAAAAAAGGG CGGCCGCGAC TCTAGAGTCG  
AGGATTACAT TTACGAATA AATAAATAA CCCGATGTAA CATTCTAGGT AGATGTTTTT TTTTTTTTTT TTTTTTTCCC GCCGGCGCTG AGATCTCAGC  
568 ProAsnVal AsnAlaLeuP heIleTyrLe uGlyTyrile ValArgSerI letyrLysly sLysLysLys LysLysArgA laAlaAlaTh rLeuGluSer  
LeuMetOC \*MetLeuTyr LeuPheIleT rpAlaThrLe uOC\*AspPro SerThrLysL ysLysLysly sLysLysGly ArgProArgL euAM\*SerArg  
SerOC\*CysL ysCysPheIl etyrLeuPhe GlyLeuHisC ysLysIleHi sLeuGlnLys LysLysLysL ysLysLysG l yGlyArgAsp SerArgValAsp  
1801 ACCTGCAGAA GCTTGGCCGC CATGGCC  
TGGACGTCTT CGAACCGCGG GTACCGG  
601 ThrCysArgS erLeuAlaAl aMetAla  
ProAlaGlu AlaTrpProp roTrp  
LeuGlnly sleuGlyArg HisGly

The opinion in support of the decision being entered today is not binding precedent of the Board.



Paper 95

By: Trial Section Merits Panel  
Board of Patent Appeals and Interferences  
Mail Stop Interference  
P.O. Box 1450  
Alexandria, VA 22313-1450  
Tel: 571-272-9797  
Fax: 571-273-0042

Filed: March 9, 2007

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS  
AND INTERFERENCES  
(Administrative Patent Judge Richard E. Schafer)

**Human Genome Sciences, Inc.,**  
Junior Party  
(Application 09/042,583-IFW  
Inventors: JIAN NI, REINER L. GENTZ,  
GUO-LIANG YU and CRAIG A. ROSEN),

v.

**Immunex Corp.,**  
Senior Party  
(Patent 6,569,642  
Inventors: CHARLES RAUCH and HENNING WALCZAK).

Patent Interference No. 105,380 (RES)

Before: SCHAFFER, HANLON and SPIEGEL, Administrative Patent Judges.

SPIEGEL, Administrative Patent Judge.

**DECISION - MOTIONS - Bd.R. 125(a)**

**ADE- 37**  
USSN 10/052,798

1    **I.     Introduction**

2           This is a decision on the motions remaining in interference 105,380.

3           Junior party Ni has filed four motions, one of which has been previously  
4   decided.<sup>1</sup> Senior party Rauch has filed five motions.

5           Ni substantive motion 1 to substitute proposed count 2 for Count 1 is  
6   **denied**. Ni substantive motion 2 to be accorded priority benefit of the 17 March  
7   1997 and 29 July 1997 filing dates of its U.S. provisional applications 60/040,846  
8   and 60/054,021, respectively, is **dismissed** as moot as to Ni's proposed count 2,  
9   **granted** as to application 60/054,021 for Count 1, and **denied** as to application  
10   60/040,846. Ni miscellaneous motion 4 to exclude certain evidence is **denied**.

11          Rauch substantive motion 1 to be accorded priority benefit of the 4 June  
12   1997 and 28 March 1997 filing dates of its U.S. applications 08/869,852 and  
13   08/829,536, respectively, as to Count 1 is **granted**. Rauch substantive motion 2  
14   to designate Ni claims 321, 322, 324 and 477 of the '583 application as  
15   corresponding to Count 1 is **denied**. Rauch substantive motion 3 for judgment  
16   that Ni claims 287, 289-299, 351-361, 389-403, 431, 432, 434-442, 446, 448-  
17   458, 507-517, 581 and 623-632 are unpatentable under 35 U.S.C. §§ 102(a)  
18   and/or (e) over any one of U.S. Patents 6,642,358, 6,072,047 and 6,569,642 and  
19   WO 98/35986 is **granted to the extent** that the claims 287, 289-299, 351-361,  
20   389-403, 431, 432, 434-442, 446, 448-458, 507-517, 581 and 623-632 are  
21   unpatentable under § 102(e) over U.S. Patent 6,072,047. Rauch responsive  
22   motion 4 for priority benefit as to Ni's proposed count 2 is **dismissed** as moot.

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<sup>1</sup> Ni substantive motion 3 (Paper 30) to hold each of Rauch's involved claims unpatentable under 35 U.S.C. § 102(e) as anticipated by U.S. Patent 6,872,568 has already been denied (Paper 46).

1 Rauch miscellaneous motion 5 to exclude certain evidence is **dismissed** as  
2 moot.

3 **II. Findings of Fact (FF)**

4 The following findings of fact are supported by a preponderance of the  
5 evidence.

- 6 1. The junior party is Jian NI, Reiner L. GENTZ, Guo-Liang YU and Craig A.  
7 ROSEN ("Ni").
- 8 2. Ni is involved in the interference on the basis of application 09/042,583  
9 ("the '583 application," NX 2024), filed 17 March 1998.
- 10 3. Ni's real party-in-interest is Human Genome Sciences, Inc. ("HGS").
- 11 4. The senior party is Charles RAUCH and Henning WALCZAK ("**Rauch**").
- 12 5. Rauch is involved in the interference on the basis of U.S. Patent  
13 6,569,642 ("the '642 patent," RX 1008), issued 27 May 2003, based on  
14 application 09/536,201 ("the '201 application"), filed 27 March 2000.
- 15 6. The '201 application has been accorded benefit for the purpose of priority  
16 of the 26 June 1997 filing date of application 08/883,036, which issued 6  
17 June 2000 as U.S. Patent 6,072,047 ("the '047 patent," RX 1048).
- 18 7. Rauch's real party-in-interest is Immunex Corp. ("Immunex").
- 19 8. The subject matter of the interference is defined by one count.
- 20 9. Count 1 is "Claim 5 of U.S. Patent 6,569,642" (Paper 1, p. 3).
- 21 10. Claim 5 of the '642 patent, written in independent form, reads:  
22 An isolated TRAIL-R DNA, wherein said DNA  
23 encodes a polypeptide comprising an amino acid  
24 sequence that is at least 90% identical to the amino  
25 acid sequence present in SEQ ID NO:2, wherein said  
26 polypeptide binds TRAIL.

11. According to the '642 patent, SEQ ID NO:2 is the 440 amino acid sequence of a full length human receptor protein (including the N-terminal signal peptide), "TRAIL-R," encoded by the DNA of SEQ ID NO:1 (RX 1008, c. 1, l. 66 - c. 2, l. 2 and c. 22, ll. 12-14).

12. The claims of the parties are:

Ni	287-299, 319-322, 324, 326-339, 351-361, 389-403, 431-432, 434-442, 446-458, 476-491, 507-517, 553-596, 598-607 and 623-632
Rauch	1-52

13. The claims of the parties which correspond to Count 1 are:

Ni	287, 289-299, 351-361, 389-403, 431-432, 434-442, 446, 448-458, 507-517, 580-596, 598-607 and 623-632
Rauch	1-5, 7-13, 15-16, 18, 22, 25-26, 28-29, 31, 35, 38-42, 44, 48 and 51

14. The claims of the parties which do not correspond to Count 1, and therefore are not part of this interference, are:

Ni	288, 319-322, 324, 326-339, 447, 476-491 and 553-579
Rauch	6, 14, 17, 19-21, 23-24, 27, 30, 32-34, 36-37, 43, 45-47, 49-50 and 52

Other findings of fact follow below.

### III. Ni Substantive Motion 1

Pursuant to 37 CFR § 41.121(a)(1)(i), Ni moves to redefine the scope of the interference by substituting proposed count 2 for current Count 1 (Paper 28). Rauch opposes (Paper 52); Ni replies (Paper 58).

15. Ni's proposed count 2 reads (Paper 28, p. 1, ¶ 1):

An isolated TRAIL-R DNA, wherein said DNA encodes a polypeptide comprising an amino acid sequence that is at least 90% identical to the amino

1 acid sequence presented in SEQ ID NO:2, wherein  
2 said polypeptide binds TRAIL or induces apoptosis.

3 16. According to Ni, its proposed count 2 simply incorporates Rauch claims 4  
4 and 5, as does the current count, and adds the language "or induces  
5 apoptosis" (*id.*).

6 It is our understanding that the source of SEQ ID NO:2 in Ni's proposed  
7 count 2 is the involved '642 patent of Rauch. With this understanding, we now  
8 address Ni motion 1.

9 17. Ni argues that the abilities to bind TRAIL and to induce apoptosis are  
10 inherent properties of the polypeptide encoded by the DNA of Count 1,  
11 although only the former is expressly recited in the count (*id.*, p. 8, ¶ 1).

12 A party seeking to change the count in an interference must demonstrate a  
13 genuine need to change the count. As stated in Louis v. Okada, 59 USPQ2d  
14 1073, 1076 (Bd. Pat. App. & Int. 2001),

15 [a]t a minimum, ... a preliminary motion to broaden out  
16 the count on the basis that a party's best or earliest proofs  
17 are outside of the current count (1) should make a proffer of  
18 the party's best proofs, (2) show that such best proofs  
19 indeed lie outside of the scope of the current count, and (3)  
20 further show that the proposed new count is not excessively  
21 broad with respect to what the party needs for its best  
22 proofs.

23 Ni seeks to change the count by adding the limitation "or induces  
24 apoptosis" as an alternative to the limitation "binds TRAIL" (FF 15). Ni seeks to  
25 change the current count because its best proofs do not explicitly recite that the  
26 TRAIL-R DNA of the count encodes a polypeptide that binds TRAIL (FF 17).  
27 However, the fact that Ni's "best proofs" do not explicitly recite the language of

1 the count does not alone establish that those proofs are not directed to "subject  
2 matter" defined by the count. "The invention is not the language of the count but  
3 the subject matter thereby defined." Silvestri v. Grant, 496 F.2d 593, 598, 181  
4 USPQ 706, 709 (CCPA 1974). In appropriate circumstances, express limitations  
5 of the count may be shown to be inherent in the proofs. Id. ("In reaching this  
6 conclusion, we do not disregard the fact that the count also requires that the  
7 ampicillin possesses greater storage-stability than hydrated ampicillin and have a  
8 molecular weight of about 349. However, we regard these as inherent properties  
9 of Form II ampicillin which add nothing to the count definition beyond that  
10 determined by the [other limitations].").<sup>2</sup> The limitation said not to be disclosed  
11 by Ni's best proofs, i.e., the ability to bind TRAIL, may be shown to be an  
12 inherent property of the polypeptide encoded by the DNA of the count. In fact, Ni  
13 argues that both the ability to bind TRAIL and the ability to induce apoptosis are  
14 both inherent properties of the polypeptides encoded by the DNA of the count:

15           The ability to bind TRAIL is an expressly recited  
16           property of the polypeptides encoded by the DNA and  
17           it is an inherent property of the polypeptide of SEQ ID  
18           NO:2. Similarly, the ability of the polypeptide of SEQ  
19           ID NO:2 to induce apoptosis is also an inherent  
20           property of the polypeptide of SEQ ID NO:2.

21 [Paper 28, p. 8 (citation to material facts omitted).] Additionally, Ni has not  
22 asserted that there are any DNA molecules which would express polypeptides

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<sup>2</sup> In Silvestri, the count was directed to a new crystalline form of ampicillin which was "substantially free of water in the chemically bound state" and had a molecular weight of about 349, a particular infrared ("IR") spectrograph and improved storage stability vis-à-vis the previously known form of ampicillin. Id., 496 F.2d at 595-96, 181 USPQ at 709-710. The court held that it was sufficient to possess the claimed compound and to characterize it by its water content and IR spectrograph, without demonstrating knowledge of the ampicillin's molecular weight because the molecular weight "add[s] nothing to the count beyond that determined by the water content and infrared spectrograph." Id., 496 F.2d at 599, 181 USPQ at 709.

1 meeting the amino acid sequence requirement of the count which would induce  
2 apoptosis, but not bind TRAIL. Consequently, adding the phrase "or induces  
3 apoptosis" to Count 1 has not been shown to be necessary to encompass Ni's  
4 best proofs. Furthermore, changing the scope of the count would leave Ni in  
5 essentially the same position it is in now of having to prove an inherent property  
6 of the receptor polypeptide encoded by the DNA of the count (FF 17). Hence, Ni  
7 has failed to demonstrate that its best proofs are outside the scope of the current  
8 count and, therefore, that there is a genuine need to change the count.

9 Based on the foregoing, Ni substantive motion 1 is **denied**.

10 **IV. Rauch Responsive Motion 4**

11 Pursuant to 37 CFR § 41.121(a)(2), Rauch moves to be accorded benefit  
12 for the purpose of priority of the 26 June 1997, 4 June 1997 and 28 March 1997  
13 filing dates of U.S. applications 08/883,036, 08/869,852 and 08/829,536,  
14 respectively, as to Ni proposed count 2 (Paper 44). Rauch responsive motion 4  
15 is contingent upon the grant of Ni substantive motion 1 to substitute Ni proposed  
16 count 2 for current Count 1. Since the contingency has not occurred, Rauch  
17 responsive motion 4 is **dismissed** as moot.

18 **V. Rauch Substantive Motion 1**

19 Pursuant to 37 CFR § 41.121(a)(1)(ii), Rauch moves to be accorded  
20 benefit for the purpose of priority of the 4 June 1997 and 28 March 1997 filing  
21 dates of U.S. applications 08/869,852 ("the '852 application," RX 1017) and  
22 08/829,536 ("the '536 application," RX 1016), respectively, as to Count 1 (Paper  
23 33). Ni opposes (Paper 48); Rauch replies (Paper 60).



1           The '201 application from which Rauch's involved '642 patent issued has  
2           already been accorded benefit of the 26 June 1997 filing date of Rauch's earlier  
3           filed '036 application (FF 6).

4           18. The '201 application is a continuation of the '036 application.

5           19. The disclosures of the '201 and '036 applications are substantially  
6           identical.

7           20. The '036 application is a continuation-in-part of the '852 application,  
8           which is a continuation-in-part of the '536 application.

9           21. The '852 application was filed 4 June 1997 (RX 1017, p. 55).

10          22. The '536 application was filed 28 March 1997 (RX 1016, p. 32).

11          To be accorded benefit for the purpose of priority in an interference  
12          proceeding "means Board recognition that a patent application provides a proper  
13          constructive reduction to practice under 35 U.S.C. 102(g)(1)." 37 CFR § 41.201.  
14          A constructive reduction to practice "means a described and enabled anticipation  
15          under 35 U.S.C. 102(g)(1) in a patent application of the subject matter of a  
16          count." Id. Benefit for the purpose of priority focuses on the subject matter of a  
17          count and only requires a constructive reduction to practice of a single  
18          embodiment within the scope of the count. Falkner v. Inglis, 463 F.3d 1376,  
19          1379, 79 USPQ2d 1001, 1004 (Fed. Cir. 2006); Hunt v. Treppschuh, 523 F.2d  
20          1386, 1389, 187 USPQ 426, 429 (CCPA 1975).<sup>3</sup>

21          The subject matter of Count 1 involves isolated DNA encoding a polypeptide  
22          having an amino acid sequence which (a) is at least 90% identical to SEQ ID

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<sup>3</sup> In contrast, benefit for the purpose of 35 U.S.C. § 120 and related statutes focuses on the subject matter of the claim and requires the application for which benefit is sought to describe and enable the entire scope of the claim.

- 1 NO:2 of the '642 patent and (b) binds TRAIL (FF 10). SEQ ID NO:2 of the '642  
2 patent is the amino acid sequence of a full-length human receptor protein called  
3 TRAIL-R and is encoded by the DNA of SEQ ID NO:1 in the '642 patent (FF 11).
- 4 23. It is undisputed that the TRAIL-R protein described in SEQ ID NO:2 of  
5 the involved '642 patent is the 440 amino acid isoform of a receptor  
6 protein alternatively referred to in the literature as TR-2, DR5, Apo-2,  
7 TRICK2 and KILLER (see Paper 48, p. B-1 where Ni admits Rauch  
8 Statement of Material Facts ("SMFs") 1 and 6 as set forth in Paper 33, p.  
9 10).<sup>4</sup>
- 10 24. According to the '642 specification, TRAIL or "TNF-related apoptosis-  
11 inducing ligand" is a member of the tumor necrosis factor ("TNF") family  
12 of ligands and TRAIL-R binds TRAIL (RX 1008, c. 1, ll. 18-20 and 60-61).
- 13 25. Further according to the '642 specification, "[c]ertain uses of TRAIL-R  
14 flow from this ability to bind TRAIL, . . . TRAIL-R finds use in inhibiting  
15 biological activities of TRAIL, or in purifying TRAIL by affinity  
16 chromatography, for example" (*id.*, c. 1, ll. 61-65; these and additional  
17 uses are set forth at c. 15, l. 41 - c. 20, l. 18).
- 18 26. Example 6 in the '642 specification (c. 27, l. 26 - c. 28, l. 24) is said to  
19 demonstrate the ability of full length human TRAIL-R to bind TRAIL.
- 20 27. It was known at the time both the earlier '852 and '536 Rauch  
21 applications were filed that TRAIL was capable of inducing apoptosis (RX

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<sup>4</sup> An isoform is one of the several forms in which a protein may exist in various tissues.

1           1026;<sup>5</sup> see also Paper 48, p. B-1 where Ni admits Rauch SMF 12 as set  
2           forth in Paper 33, p. 11).

3           28. SEQ ID NO:1 of the '852 application is said to present a DNA sequence  
4           encoding a human TRAIL receptor protein (TRAIL-R) having the amino  
5           acid sequence set forth in SEQ ID NO:2 of the '852 application (RX 1017,  
6           p. 13-15).

7           29. The '852 specification explicitly states that "[t]he present invention  
8           provides isolated nucleic acids useful in the production of TRAIL-R  
9           polypeptides, ... Such nucleic acids include, but are not limited to, the  
10          human TRAIL-R DNA of SEQ ID NO:1." (RX 1017, p. 27, ll. 26-29).

11          30. It is undisputed that the amino acid sequence described in SEQ ID NO:2  
12          of the '852 application is identical to amino acid sequence SEQ ID NO:2  
13          of the '642 patent (see Paper 48, B-1 where Ni admits Rauch SMF 10 as  
14          set forth in Paper 33, p. 11).

15          31. According to the '852 specification, TRAIL-R binds to the cytokine TRAIL  
16          and "[c]ertain uses of TRAIL-R flow from this ability to bind TRAIL, . . . .  
17          TRAIL-R finds use in inhibiting biological activities of TRAIL, or in  
18          purifying TRAIL by affinity chromatography, for example" (RX 1017, p. 2,  
19          ll. 8-12; these and additional uses of TRAIL-R are set forth at p. 20, l. 15 -  
20          p. 25, l. 14).

21          32. Example 6 in the '852 specification (p. 35, l. 4 - p. 36, l. 13) is said to  
22          demonstrate the ability of full length human TRAIL-R to bind TRAIL.

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<sup>5</sup> Wiley et al., "Identification and Characterization of a New Member of the TNF Family that Induces Apoptosis," Immunity, Vol. 3, pp. 673-682 (December 1995) (RX 1026).

- 1        33. Thus, the '852 application describes an embodiment within the scope of
- 2            Count 1, i.e., a DNA sequence encoding a human TRAIL receptor protein
- 3            (TRAIL-R) having an amino acid sequence identical to the amino acid
- 4            sequence of SEQ ID NO:2 of the '642 patent (FFs 28-30) and which
- 5            binds TRAIL (FFs 31-32).
- 6        34. Ni does not dispute Rauch's claim to benefit for the purpose of priority of
- 7            the filing date of its '852 application (Paper 48).
- 8        35. Figure 2 of the '536 application is said to present a DNA sequence
- 9            encoding a human TRAIL receptor protein (TRAIL-R) having the amino
- 10           acid sequence set forth in Figure 3 of the '536 application (RX 1016, p. 2,
- 11           II. 1-5).
- 12       36. The '536 specification explicitly states that "TRAIL-R DNA may be used
- 13           to prepare TRAIL-R polypeptides encoded by the DNA" (RX 1016, p. 6, II.
- 14           7-10).
- 15       37. It is undisputed that the nucleic acid and amino acid sequences of the
- 16           DNA and encoded TRAIL-R protein described in Figures 2 and 3 of the
- 17           '536 application are identical to the nucleic acid and amino acid
- 18           sequences described in SEQ ID NOs: 1 and 2, respectively, of the '642
- 19           patent (see Paper 48, p. B-1 where Ni admits Rauch SMFs 13 and 14 as
- 20           set forth in Paper 33, pp. 11-12).
- 21       38. According to the '536 specification, TRAIL-R binds to the cytokine TRAIL
- 22           and "[c]ertain uses of TRAIL-R flow from this ability to bind TRAIL, . . . .
- 23           TRAIL-R finds use in inhibiting biological activities of TRAIL, or in

- 1           purifying TRAIL by affinity chromatography, for example" (RX 1016, p. 2,  
2           II. 8-12; these and additional uses are set forth at p. 13, I. 34 - p. 18, I.  
3           26).
- 4           39. Thus, the '536 application describes an embodiment within the scope of  
5           Count 1, i.e., a DNA sequence encoding a human TRAIL receptor protein  
6           (TRAIL-R) having an amino acid sequence identical to the amino acid  
7           sequence of SEQ ID NO:2 of the '642 patent (FFs 35-37) and which  
8           binds TRAIL (FF 38).
- 9           40. Ni disputes Rauch's claim to benefit of the filing date of the '536  
10           application, contending that the '536 application fails to show any utility  
11           for the DNA molecule and, therefore, fails the "how to use" of the  
12           enablement requirement (Paper 48, § C.2, pp. 5-6).
- 13           41. Ni relies on Rasmusson v. SmithKline Beecham Corp., 413 F.3d 1318,  
14           75 USPQ2d 1297 (Fed. Cir. 2005) to support its conclusion that  
15           a party cannot establish that an earlier application  
16           constitutes a constructive reduction to practice without  
17           at the very least showing that the earlier application  
18           discloses a utility for an embodiment of the count. In  
19           other words, an essential element of a party's case for  
20           benefit of an earlier application is a demonstration  
21           that the earlier application satisfies the how-to-use  
22           prong of § 112, first paragraph, with respect to at least  
23           one embodiment of the count. [Paper 48, p. 5, ¶ 2.]
- 24           42. Specifically, Ni argues that "Rauch has neglected to assert, implicitly or  
25           explicitly, that the '536 application discloses any utility for a DNA  
26           molecule within the scope of the count" (Paper 48, p. 7, ¶ 3).

1 In essence, the only opposition raised by Ni is whether the '536 application  
2 discloses an adequate utility/enablement for a DNA embodiment within the scope  
3 of Count 1. First, count 1 explicitly describes a utility for a DNA embodiment  
4 within the scope of Count 1, i.e., it encodes a polypeptide having an amino acid  
5 sequence which is at least 90% identical to SEQ ID NO:2 of the '642 patent and  
6 binds TRAIL. Second, Rauch asserted this utility/enablement (Paper 33, pp. 5-6)  
7 and pointed to express descriptive support of an embodiment within the scope of  
8 Count 1 in the '536 application in Appendix C of its motion, i.e., "Figure 2  
9 discloses a DNA sequence that encodes the polypeptide set forth in Figure 3"  
10 (Paper 33, p. 13, c. 2). Third, the '536 specification explicitly states that "TRAIL-  
11 R DNA may be used to prepare TRAIL-R polypeptides encoded by the DNA" (FF  
12 36). Fourth, our finding that the '536 specification describes and enables an  
13 embodiment within the scope of Count 1 is not inconsistent with the holding in  
14 Rasmusson.

15 In Rasmusson, both parties had interfering claims directed to methods of  
16 treating prostate cancer comprising administering finasteride, a selective 5- $\alpha$ -  
17 reductase inhibitor. An interference was declared by the Board of Patent  
18 Appeals and Interferences ("the Board"). Rasmusson was involved in the  
19 interference on the basis of an application which claimed priority to eight earlier  
20 filed applications. SmithKline Beecham Corp. was involved in the interference on  
21 the basis of two patents and corresponding reissue applications. On appeal from  
22 the decision of the Board, the Federal Circuit affirmed the Board's holding that  
23 Rasmusson was not entitled to benefit for the purpose of priority of the filing

1 dates of the eight earlier filed applications. Citing In re Brana, 51 F.3d 1560, 34  
2 USPQ2d 1436 (Fed. Cir. 1995), the court said "a specification disclosure which  
3 contains a teaching of the manner and process of making and using the invention  
4 . . . must be taken as in compliance with the enabling requirement of the first  
5 paragraph of § 112 unless there is reason to doubt the objective truth of the  
6 statements contained therein which must be relied on for enabling support"  
7 (Rasmusson, 413 F.3d at 1323, 75 USPQ2d at 1300, emphasis added). The  
8 court affirmed the Board's finding that one of ordinary skill in the art would not  
9 have believed that finasteride was effective in treating prostate cancer in light of  
10 the state of the art at the relevant time and because Rasmusson had failed to  
11 provide experimental proof demonstrating the effectiveness of the invention (id.,  
12 413 F.3d at 1324-25, 75 USPQ2d at 1301).

13 Here, the '536 specification explicitly states that "TRAIL-R DNA may be  
14 used to prepare TRAIL-R polypeptides encoded by the DNA" (FF 36). The '536  
15 specification further describes certain uses of TRAIL-R based on its ability to bind  
16 TRAIL, e.g., using TRAIL-R to purify TRAIL by affinity chromatography (FF 38).  
17 Ni has not pointed to evidence of record which raises doubts as to the objective  
18 truth of these statements in the '536 specification, as was the case in  
19 Rasmusson. For example, Ni does not allege or provide evidence that one of  
20 ordinary skill in the art could not use a DNA to produce the protein encoded  
21 thereby or that a receptor protein that binds a ligand could not be used to purify  
22 the ligand using affinity chromatography at the time the '536 application was filed.  
23 Specifically, Ni has not shown that Rauch failed to satisfy its burden of proof with

1 respect to enablement regarding the DNA embodiment of the count set forth in  
2 Figure 2 of the '536 application, i.e., that the DNA of Figure 2 did not encode, and  
3 therefore was not useful to produce, the TRAIL-R polypeptide set forth in Figure  
4 3. Moreover, Ni does not argue that the '536 application fails to disclose any  
5 utility for the TRAIL-R polypeptide set forth in its Figure 3. In short, Rauch  
6 described how to use a DNA within the scope of Count 1, i.e., the DNA of Figure  
7 2 encodes a protein that binds TRAIL (Paper 33, pp. 5-6), and Ni has not  
8 asserted that encoding a protein that binds TRAIL is not a sufficient utility nor  
9 provided any basis to doubt the objective truth of express statements in the '536  
10 specification that the DNA of Figure 2 is useful to produce the encoded TRAIL-R  
11 polypeptide.

12 Based on the foregoing, Rauch substantive motion 1 is **granted**.

13 **VI. Ni Substantive Motion 2**

14 Pursuant to 37 CFR § 41.121(a)(1)(ii), Ni moves to be accorded benefit for  
15 the purpose of priority of the 17 March 1997 and 29 July 1997 filing dates of its  
16 earlier provisional applications 60/040,846 ("the '846 application," NX 2042) and  
17 60/054,021 ("the '021 application," NX 2056), respectively, as to Count 1 and,  
18 contingent on the grant of Ni substantive motion 1, as to Ni's proposed count 2  
19 (Paper 29). Rauch opposes (Paper 53); Ni replies (Paper 59).

20 To the extent Ni substantive motion 2 is contingent upon the grant of Ni  
21 substantive motion 1, it is **dismissed** as moot because the contingency has not  
22 occurred.



1           As discussed above, the subject matter of Count 1 is directed to isolated  
2 DNA that encodes a polypeptide having an amino acid sequence that is at least  
3 90% identical to SEQ ID NO:2 of Rauch's involved '642 patent, wherein the  
4 polypeptide binds TRAIL (FF 10 and 11). TRAIL is a member of the TNF ligand  
5 family and was known to be capable of inducing apoptosis (FF 27). To be  
6 accorded benefit of the filing date of an earlier filed application, the earlier  
7 application must provide a constructive reduction to practice of an embodiment  
8 within the count, i.e., a described and enabled anticipation of the subject matter  
9 of the count.

10           43. The '021 and '846 applications are both provisional applications.

11           44. The '021 application was filed 29 July 1997 (NX 2056, cover sheet).

12           45. The '846 application was filed 17 March 1997 (NX 2042, cover sheet).

13           46. Figure 1 of the '021 application is said to show the nucleotide and  
14 deduced amino acid sequences of "human Death Domain Containing  
15 Receptor 5" ("DR5") obtained from the cDNA clone deposited as ATCC  
16 Deposit No. 97920 on 7 March 1997 (NX 2056, p. 1, ll. 7-9; p. 6, ll. 5-6; p.  
17 7, ll. 29-33; p. 9, ll. 9-12; p. 10, ll. 34-35).

18           47. According to the '021 specification, DR5 is a 411 amino acid protein (id.,  
19 p. 26, ll. 9-10).

20           48. Example 6 of the '021 specification is said to show that a DR5  
21 extracellular domain-Fc fusion construct ("DR5-Fc") binds TRAIL (id., p.  
22 50, l. 6 - p. 51, l. 2; Figures 6A-6C).

- 1        49. Figure 1 of the '846 application is said to show the nucleotide and  
2        deduced amino acid sequences of DR5 obtained from the cDNA clone  
3        deposited as ATCC Deposit No. 97920 on 17 March 1997 (NX 2042, p.  
4        1, ll. 5-6; 3, ll. 22-25; p. 5, ll. 24-27).
- 5        50. According to the '846 specification, DR5 is a 411 amino acid protein (id.,  
6        p. 6, ll. 25-27).
- 7        51. Figure 2 of the '846 application is said to compare the deduced amino  
8        acid sequence of DR5 to the amino acid sequences of human tumor  
9        necrosis factor 1, human Fas protein and DR3 protein (id., p. 5, ll. 8-13).
- 10       52. According to the '846 specification, similarities between the amino acid  
11       sequences shown in Figure 2 "**strongly suggests** that DR5 is also a  
12       death domain containing receptor with the ability to induce apoptosis,"  
13       i.e., that DR5 belongs to a subset of TNF-family receptors (id., p. 6, ll. 31-  
14       33, emphasis added).
- 15       53. Further according to the '846 specification, "TNF-family ligands induce  
16       various cellular responses by binding to TNF-family receptors, including  
17       the DR5 of the present invention. Cells which express the DR5  
18       polypeptide **are believed to have** a potent cellular response to DR5  
19       ligands ..." (NX 2042, p. 26, ll. 12-15, emphasis added).
- 20       54. The '846 specification defines a "TNF-family ligand" as a  
21       naturally occurring, recombinant, and synthetic  
22       ligands that are capable of binding to a member of the  
23       TNF receptor family and inducing the ligand/receptor  
24       signaling pathway. Members of the TNF ligand family  
25       include, but are not limited to, **DR5 ligands**, TRAIL,  
26       TNF- $\alpha$ , lymphdotoxin- $\alpha$  [sic] (LT- $\alpha$ , also known as TNF-  
27        $\beta$ ), LT- $\beta$  (found in complex heterotrimer LT- $\alpha$ 2- $\beta$ ),

1 FasL, CD40, CD27, CD30, 4-1BB, OX40 and nerve  
2 growth factor (NGF). [NX 2042, p. 31, ll. 4-9,  
3 emphasis added.]

4 55. The amino acid sequence of the DR5 protein shown in the respective  
5 Figures 1 of the '021 and '846 applications are identical.

6 56. It is undisputed that the amino acid sequence shown in Figures 1 of the  
7 '021 and '846 applications are at least about 93% identical to the amino  
8 acid sequence of SEQ ID NO:2 as recited in Count 1, with 411 of 440  
9 total amino acids being identical (see Paper 53, p. 23 where Rauch  
10 admits Ni SMFs 7 and 8).

11 57. Thus, the '021 application describes an enabled embodiment within the  
12 scope of Count 1, i.e., a DNA sequence encoding a human Death  
13 Domain Containing Receptor 5 ("DR5") having an amino acid sequence  
14 that is at least 93% identical to the amino acid sequence of SEQ ID NO:2  
15 of the '642 patent (FFs 46, 47 and 56) and which binds TRAIL (FF 48).

16 58. Rauch does not dispute Ni's claim to benefit for the purpose of priority of  
17 the filing date of its '021 application (Paper 53).

18 Based on the foregoing, we accord Ni benefit of the filing date of the '021  
19 application as to Count 1.

20 While the '846 specification describes (Figure 1) an isolated DNA  
21 encoding polypeptide DR5 comprising a deduced amino acid sequence which is  
22 at least 90% identical to the amino acid sequence set forth in SEQ ID NO:2 of the  
23 '642 patent (411 of 440 amino acids are identical), the disclosure of the '846  
24 application simply suggests that the DR5 polypeptide encoded by the isolated  
25 DNA is a death domain containing receptor with the ability to induce apoptosis

- 1 and suggests that the DR5 polypeptide binds a "DR5 ligand" (FFs 52 and 53).
- 2 The disclosure of the '846 application does not describe preparing a DR5
- 3 polypeptide (or ligand binding portion thereof) or binding the ligand TRAIL to the
- 4 DR5 polypeptide (or ligand binding portion thereof).
- 5 Ni's position is premised on classifying DR5 as a putative TNF "death
- 6 receptor" protein based on the described similarity between DR5 and three
- 7 previously known TNF death receptors TNFR1, Fas and DR3 in the '846
- 8 application. According to Ni, TNFR1, Fas and DR3 were all known to induce
- 9 apoptosis upon activation and, therefore, that same function should be imputed
- 10 to DR5 by virtue of the described similarities between the amino acid sequences
- 11 of DR5 and the three death receptors. Ni argues that the '846 specification
- 12 explicitly teaches that DR5 induces apoptosis and binds to a TNF ligand selected
- 13 from a limited list including TRAIL. Ni further argues that, based on the doctrine
- 14 of inherency, the '846 application need not expressly recite that DR5 binds TRAIL
- 15 so long as the '846 application describes the subject matter of the count. [Paper
- 16 29, p. 2, ¶ 3 and ¶ bridging pp. 9-10.]
- 17 59. Ni relies on the direct testimony of John C. Reed, M.D., Ph.D. (NX 2099)
- 18 in support of its position.
- 19 60. Dr. Reed has been qualified as an expert to give opinions on the subjects
- 20 of apoptosis and of the tumor necrosis family of ligands (TNFs) and
- 21 receptors (TNFRs), including death receptors.
- 22 61. According to Dr. Reed, the deduced amino acid sequence of human DR5
- 23 described in the '846 application has all the canonical (structural)

- 1 features of a classic death receptor of the TNFR family, i.e., a leader  
2 peptide, conserved cysteine-rich domain(s), a transmembrane domain  
3 and a cytosolic domain containing a "death domain" (NX 2099, ¶¶ 19-20  
4 and 28-31).
- 5 62. Further according to Dr. Reed, the death domain "is necessary and  
6 sufficient for apoptosis induction, at least when overexpressed in  
7 mammalian cells" (NX 2099, ¶ 21).
- 8 63. Still further according to Dr. Reed, DR5 shares the highest degree of  
9 amino acid sequence identity with then known death receptor proteins  
10 human TNFR1, Fas and DR3 (NX 2099, ¶¶ 20 and 29).
- 11 64. Dr. Reed states that the deduced amino acid sequence of the "death  
12 domain" region of the DR5 protein described in Ni's '846 application was  
13 approximately 21, 32 and 33 percent identical to the amino acid  
14 sequences of the death domains of known death receptors Fas, TNFR1  
15 and DR3, respectively, "using Lipman-Pearson Protein Alignment (with  
16 the following parameters: Ktuple 2; Gap Penalty 4; Gap Length Penalty  
17 12)" (NX 2099, ¶ 31).
- 18 65. Dr. Reed opines that a death domain amino acid sequence identity of  
19 approximately 21-33 percent is "significant" because Chinnaniyan (NX  
20 2058) reported that the death domain of DR3 was 47 and 23 percent  
21 identical to that of TNFR1 and Fas, respectively, while Marsters (NX  
22 2059) reported that the death domain of DR3 was 48 and 20 percent  
23 identical to that of TNFR1 and Fas, respectively (NX 2099, ¶ 31).

- 1        66. Chinnaiyan reported using MegAlign™ software to align the compared  
2        amino acid sequences (NX 2058, Fig. 1).
- 3        67. MegAlign™ software can create alignments between two or more  
4        sequences according to different methods, e.g., the clustal method or the  
5        Jotun Hein method (see e.g., U.S. Patent 6,277,568, col. 8, ll. 22-41).
- 6        68. Neither Chinnayian nor Marsters reported the alignment program and  
7        parameters used to obtain their respective percent sequence identity  
8        scores.
- 9        69. Dr. Reed did not explain percent sequence identity scoring, e.g., how  
10       different alignment methods and parameters calculate percent sequence  
11       identity scores; how different alignment methods are compared  
12       (normalized to account for the use of parameter differences in sequence  
13       lengths, gaps, gap positions, etc.); the significance, if any, of comparing  
14       sequences within predicted structural features (e.g., a death domain or  
15       extracellular domain) versus over their entire primary amino acid  
16       sequence; standard error of the method(s) used; use of iteration, etc.
- 17       70. For example, according to Tartaglia,  
18           [i]t has been noted previously that the intracellular  
19           domain of TNF-R1 shares a weak homology (29%  
20           identity over 45 amino acids) with the intracellular  
21           domain of Fas antigen. Upon further inspection of  
22           these sequences, we noted that introduction of a 1  
23           amino acid gap in the Fas sequence extended the  
24           region of homology an additional 20 amino acids  
25           (Figure 3). [NX 2067,<sup>6</sup> p. 846, col. 2, ¶ 1, emphasis  
26           added, citation omitted.]

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<sup>6</sup> Tartaglia et al. (Tartaglia), "A Novel Domain within the 55 kd TNF Receptor Signals Cell Death," Cell, Vol. 74, pp. 845-853 (10 September 1993) (NX 2067).

1       71. Nonetheless, Dr. Reed believes that one of ordinary skill in the art would  
2       have reasonably expected the putative death receptor DR5 of the '846  
3       specification to have utilities similar to known utilities of other known  
4       death receptors TNFR1, Fas and DR3 (NX 2099, ¶¶ 33-34).

5       72. According to Dr. Reed, "**the most reasonable conclusion to draw** from  
6       Ni's March 17, 1997 application is that DR5 is expected, by persons of  
7       ordinary skill in the art, to be a novel death receptor" and, therefore,  
8       skilled artisans "**would have predicted** that activation of DR5 would  
9       induce apoptosis" (NX 2099, ¶ 32, emphasis added).

10      73. According to Dr. Reed, induction of apoptosis involves activating  
11      (aggregating) the death receptor on the surface of a cell (in its  
12      membrane) and activating a family of caspase enzymes inside the cell  
13      (NX 2099, ¶ 24).

14      74. Further according to Dr. Reed, activation (aggregation) of the death  
15      receptor could be caused by (i) ligand binding to the death receptor, (ii)  
16      antibody binding to the death receptor or (iii) overexpression of the death  
17      receptor on the cell surface (NX 2099, ¶ 24).

18      75. Dr. Reed testified that  
19          if one would want to determine to which TNF ligand  
20          DR5 binds, Ni's March 17, 1997 application [i.e., the  
21          '846 application], in combination with what was known  
22          in the art at the time, provides all of the necessary  
23          information. For example, Ni's March 17, 1997  
24          application states that **DR5 binds to a TNF-family**  
25          **ligand** (Exhibit 2042, pg. 4, ¶¶ 2-3; pg. 26, ¶ 1; pages  
26          28-29; pg. 31, ¶ 1, pg. 31, ¶ 1 [sic]), which would have  
27          been expected by a person of ordinary skill in the art  
28          in view of the literature that was available by March  
29          17, 1997. Additionally, Ni's March 17, 1997

- 1 application specifically defines "a TNF family ligand"  
2 as a limited number of molecules, one of which is  
3 TRAIL. (Exhibit 2042, pg. 31, lines 4-9). The Ni  
4 March 17, 1997 application also teaches assays, such  
5 as cellular response assays, **that could be used to**  
6 **determine whether** TRAIL, or any other of the **listed**  
7 **TNF ligands, binds to DR5.** (Exhibit 2042, pg. 26,  
8 lines 12-26; pg. 27, line 21 through pg. 29, line 6).  
9 **Alternatively, as of March 17, 1997, it would have**  
10 **been routine** for one of ordinary skill in the art **to**  
11 **have tested whether DR5 binds to the TNF-family**  
12 **ligands recited in Ni's May [sic] 17, 1997**  
13 **application,** including TRAIL. Thus, if one wanted to  
14 have determined whether DR5 bound to a TNF  
15 ligand, including TRAIL, the Ni March 17, 1997  
16 application, in combination with what was known in  
17 the art at the time, teaches all of the needed  
18 information. [NX 2099, ¶ 56, emphasis and bracketed  
19 text added.]
- 20 76. Dr. Reed notes that while most TNF family receptors have been shown  
21 experimentally to bind to specific TNF family ligands, some receptors "do  
22 not have known ligands to date, or a delay of many years occurred  
23 before the specific ligand was established" (NX 2099, ¶ 18).
- 24 77. According to the '846 specification, there are eleven known TNF ligand  
25 members, i.e., TNF- $\alpha$ , lymphotoxin- $\alpha$  (LT- $\alpha$ , also known as TNF- $\beta$ ), LT- $\beta$   
26 (found in complex heterotrimer LT- $\alpha$ 2- $\beta$ ), FasL, CD40, CD27, CD30, 4-  
27 1BB, OX40 and nerve growth factor (NGF) (NX 2042, p. 1, ll. 21-25) and  
28 TRAIL (*id.*, p. 31, ll. 6-9).
- 29 78. TRAIL was identified as a TNF family ligand by at least December 1995  
30 (NX 2096).
- 31 79. The '846 specification defines "TNF-family ligand" as  
32 naturally occurring, recombinant, and synthetic  
33 ligands that are capable of binding to a member of the  
34 TNF receptor family and inducing the ligand/receptor



1 signaling pathway. Members of the TNF ligand family  
2 include, but are not limited to, **DR5 ligands**, TRAIL,  
3 TNF- $\alpha$ , lymphdotoxin- $\alpha$  [sic] (LT- $\alpha$ , also known as TNF-  
4  $\beta$ ), LT- $\beta$  (found in complex heterotrimer LT- $\alpha$ 2- $\beta$ ),  
5 FasL, CD40, CD27, CD30, 4-1BB, OX40 and nerve  
6 growth factor (NGF). [NX 2042, p. 31, ll. 4-9,  
7 emphasis added.]

8 80. Dr. Reed relies on Ni's later filed '201 application (NX 2056, Figure 6A)  
9 and on a later published August 1997 article (NX 2031<sup>7</sup>) to support his  
10 testimony that DR5 "necessarily" binds to TRAIL and "necessarily"  
11 induces apoptosis (NX 2099, ¶ 57).

12 A constructive reduction to practice requires a described and enabled  
13 anticipation under 35 U.S.C. § 102(g)(1). To fulfill the written description  
14 requirement, the patent specification must describe an invention in sufficient  
15 detail that one skilled in the art can clearly conclude that the inventor invented  
16 what is claimed. Lockwood v. Am. Airlines, Inc., 107 F.3d 1565, 1572, 41  
17 USPQ2d 1961, 1966 (Fed. Cir. 1997). The specification "need not describe the  
18 claimed subject matter in exactly the same terms as used in the claims; it must  
19 simply indicate to persons skilled in the art that as of the [filing] date the applicant  
20 had invented what is now claimed." Eiselstein v. Frank, 52 F.3d 1035, 1038, 34  
21 USPQ2d 1467, 1470 (Fed. Cir. 1995) (citing Vas-Cath Inc. v. Mahurkar, 935 F.2d  
22 1555, 1562, 19 USPQ2d 1111, 1115 (Fed. Cir. 1991) and In re Wertheim, 541  
23 F.2d 257, 265, 191 USPQ 90, 98 (CCPA 1976). Furthermore, "the fact that a  
24 characteristic is a necessary feature or result of a prior-art embodiment (**that is**  
25 **itself sufficiently described and enabled**) is enough for inherent anticipation,

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<sup>7</sup> Guohua Pan, Jian Ni, Ying-Fei Wei, Guo-liang Yu, Reiner Gentz, Vishva M. Dixit, "An Antagonist Decoy Receptor and a Death Domain-Containing Receptor for TRAIL," Science, Vol. 277, pp. 815-18 (8 August 1997).

1 even if that fact was unknown at the time of the prior invention" (Toro Co. v.  
2 Deere & Co., 69 USPQ2d 1584, 1590 (Fed. Cir. 2004), bold emphasis added  
3 (citing Schering Corp. v. Geneva Pharmaceuticals, Inc., 339 F.3d 1373, 1378, 67  
4 USPQ2d 1664, 1668-69 (Fed. Cir. 2003) and Atlas Powder Co. v. Ireco Inc., 190  
5 F.3d 1342, 1347, 51 USPQ2d 1943, 1947-48 (Fed. Cir. 1999)).

6 Here, the subject matter of the count is directed to a family of DNA  
7 molecules which encodes a functional protein, i.e., a TNF death receptor protein  
8 that binds TRAIL (a TNF ligand known to be capable of inducing apoptosis).  
9 Relying on the testimony of Dr. Reed, Ni contends that the similarity of the  
10 deduced amino acid sequence of DR5 to the amino acid sequences of three  
11 known TNF death receptor proteins (TNFR1, Fas and DR3) as described in the  
12 '846 application is sufficient to characterize DR5 as a putative TNF death  
13 receptor protein and to reasonably predict that DR5 has utilities/functions similar  
14 to known death receptor proteins, e.g., induction of apoptosis upon activation.

15 Neither the disclosure of the '846 application nor the testimony of Dr. Reed is  
16 as explicit as Ni argues. The '846 application suggests that the protein encoded  
17 by the DNA of Figure 1 may be classified as a putative TNF death receptor  
18 protein. Dr. Reed testified that the most reasonable conclusion a person of  
19 ordinary skill in the art would draw from the '846 application is that DR5 "is  
20 expected ... to be a novel death receptor" (FF 64). However, the factual basis for  
21 this conclusion is not persuasive. The '846 specification does not describe  
22 preparing (e.g., expressing and purifying the product of the DNA of Figure 1) a  
23 DR5 polypeptide or ligand binding portion thereof. The '846 specification does

1 not describe an activated (functional) DR5 or identify the TNF ligand which  
2 activates (binds to) DR5.

3 Since TRAIL was known to be capable of inducing apoptosis (FF 27),  
4 identifying TRAIL as the TNF ligand which bound to DR5 in the '846 application  
5 would have been one way of describing DR5 as capable of inducing apoptosis.  
6 Dr. Reed testified that the '846 application "states that DR5 binds to a TNF-family  
7 ligand" and that there were "assays, that could be used to determine whether  
8 TRAIL, or any other of the listed TNF ligands, binds to DR5" (FF 75). Dr. Reed  
9 further testified that "it would have been routine for one of ordinary skill in the art  
10 to have tested whether DR5 binds to the TNF-family ligands recited" in the '846  
11 application, "including TRAIL" (FF 75). Notably, the '846 specification  
12 enumerates "DR5 ligands" as separate and distinct ligands in the list of TNF  
13 ligands, including TRAIL (FF 54), the implication being that DR5 might bind to  
14 either a known TNF ligand, e.g., TRAIL, or an as yet unknown TNF ligand, i.e., a  
15 DR5 ligand, or another TNF ligand known to be capable of inducing another  
16 function, e.g., cell proliferation.

17 In short, there is neither explicit nor implicit disclosure in the '846 application  
18 said to show that the DR5 polypeptide encoded by the DNA of Figure 1 is a  
19 functional/bioactive protein. The cognate ligand for DR5 was not explicitly  
20 identified in the '846 application, although it would have been routine for one of  
21 ordinary skill in the art to do so using known techniques, as testified to by Dr.  
22 Reed (FF 75). However, Ni has failed to explain how such "routine  
23 experimentation" satisfies the written description requirement of 35 U.S.C. § 112,

1 first paragraph. Moreover, there could be no explicit description of an activated  
2 DR5 polypeptide based on antibody binding or overexpression in mammalian  
3 cells absent obtaining the DR5 polypeptide, e.g., by expressing (or  
4 overexpressing) the product of the DNA of Figure 1 to obtain a protein against  
5 which to raise an antibody. Furthermore, one of ordinary skill in the art could not  
6 have reasonably predicted the function(s) of DR5 based solely on the similarity  
7 between its deduced amino acid sequence as set forth in Figure 1 of the '846  
8 application and the amino acid sequences of TNFR1, Fas and DR3 in view of the  
9 state of the art when the '846 application was filed for the following reasons.

10 Genes encode proteins by providing a sequence of nucleic acids that is  
11 translated into a sequence of amino acids. Methods used to identify novel genes  
12 are classified into two types, i.e., homology based or non-homology based. In  
13 homology based methods, for example, clones from a cDNA library are cloned  
14 and analyzed (sequenced). The resultant nucleotide sequences and/or deduced  
15 amino acid sequences are checked against databases for similarity (homology)  
16 to previously characterized sequences on the theory that molecules with similar  
17 sequences would be expected to perform similar functions. However, one of the  
18 difficulties in identifying a functional protein is that function depends not only on  
19 the amino acid sequence of the protein, but also on other factors, e.g., the  
20 structure of the protein.

21 In order for a protein to function properly its amino acid sequence (primary  
22 structure) must fold itself up into a complex three-dimensional shape which  
23 allows for molecular recognition. Molecular recognition often involves only a

1 small number of key amino acid residues on the functional surfaces of interacting  
2 molecules. These residues are dispersed in diverse regions of the primary  
3 amino acid sequence due to the complex structural organization of the protein.  
4 There are multiple levels to the structural organization of a protein. The *primary*  
5 *structure* of a protein refers to the linear arrangement of amino acid residues  
6 along a polypeptide chain. *Secondary structures* form through interactions  
7 between amino acids typically found near each other in the peptide chain which  
8 fold parts of the chain into regular structures, e.g.,  $\alpha$  helices and  $\beta$  sheets.  
9 *Tertiary structure* folds both the secondary structures and the regions between  
10 them into compact three-dimensional shapes in an energetically favourable way.  
11 *Quaternary structure* refers to the organization of several polypeptide chains into  
12 a single protein molecule, e.g., hemoglobin is a tetramer. Consequently, amino  
13 acid residues rather near to each other in a protein's primary structure may be  
14 rather distant in the protein's ultimate quaternary structure. [See generally,  
15 MOLECULAR CELL BIOLOGY ("MCB"), second edition, Darnell et al., W.H.  
16 Freeman and Company, New York, NY (1990), pp. 44-48 (copy enclosed)].

17 For example, an enzyme is a protein that catalyzes a biochemical  
18 reaction. The function of an enzyme relies on the structure of its "active site," a  
19 specific cavity-like region on the surface of the three-dimensional enzyme which  
20 allows a snug fit (molecular recognition) between the enzyme and its substrate  
21 (reactant in the reaction being catalyzed). The active site contains key amino  
22 acids that bind the substrate and are involved in the reaction catalyzed by the  
23 enzyme. These key amino acids are brought into proximity (into the active site)

1 by protein folding. [See generally, MICROBIOLOGY: An Introduction, Tortora et  
2 al., The Benjamin/Cummings Publishing Company, Inc., Menlo Park, California,  
3 (1982) pp. 111-112, copy enclosed; MCB, pp. 55-65, copy enclosed.]

4 On the other hand, mutations that cause human disease often disrupt  
5 protein structure, thereby altering or abolishing normal protein function. For  
6 example, sickle cell anemia occurs in humans that are homozygous for a  $\beta$ -  
7 hemoglobin gene that differs from the normal adult hemoglobin gene by a single  
8 base pair, resulting in a change in a single amino acid from glutamate to valine in  
9 position 5. This substitution is on the surface of the abnormal hemoglobin (Hb S)  
10 and changes the electrostatic charge on the surface of Hb S. When oxygen is  
11 removed from Hb S, the protein polymerizes into rigid crystals that deform a  
12 sickle cell patient's red blood cells. Thus, although normal hemoglobin and Hb S  
13 have virtually identical primary amino acid sequences, a single amino acid  
14 change in Hb S alters its quaternary structure and results in abnormal protein  
15 function. [See generally, CLINICAL DIAGNOSIS AND MANAGEMENT BY  
16 LABORATORY METHODS, sixteenth edition, J. B. Henry ed., W.B. Saunders  
17 Company, Philadelphia (1979), Vol. I, p. 992, copy enclosed.]

18 Ergo, "[s]equence comparison can indicate whether an RNA or protein  
19 molecule or region of DNA is already known (identity) or has some degree of  
20 similarity to a known sequence" (MOLECULAR BIOLOGY AND  
21 BIOTECHNOLOGY, R. Meyers, ed., VCH Publishers, Inc., New York, NY (1995),  
22 p. 860, c. 1, ¶ 1, copy enclosed). However, since "[t]he function of nucleic acids

1 and proteins depend on their structure and involves complex interactions in three  
2 dimensions",

3 [i]t is not presently understood whether it is possible,  
4 in general, to derive structure from sequence.  
5 Sequence alone is therefore often inadequate to  
6 determine function. Predictions made from sequence  
7 analysis need to be experimentally tested.  
8 Nonetheless, computer analysis of sequences is  
9 valuable in suggesting the most useful experiments to  
10 perform. [Id., p. 860, c. 1, ¶ 2.]

11 Indeed, the difficulties in predicting the structure and function of a protein from  
12 just its amino acid sequence (primary structure) are so well known in the art that  
13 the ability to characterize the structure and function of a protein from its amino  
14 acid sequence has been called the "Holy Grail" of molecular biology (RX 1061, p.  
15 511, c.2, ¶ 1 to p. 512, c. 1, ¶ 1).

16 81. Genchong Cheng, Ph.D., is a witness for Rauch and has been qualified  
17 as an expert to give opinions on the subjects of signal transduction and  
18 gene expression networks through the TNFR, Toll-like receptor (TLR)  
19 and Nod receptor families during immune responses.

20 82. According to Dr. Cheng,

21 [s]equence homology to other death domain-  
22 containing TNF receptors may be sufficient to  
23 convince one of ordinary skill in the art that a novel  
24 protein is a TNFR family member. However,  
25 sequence homology alone is not sufficient to support  
26 an assertion that a novel TNFR family member  
27 protein will induce specific biological activities such as  
28 apoptosis. Without additional data regarding the  
29 activity of a TNFR family member, such as, for  
30 example, the identity of the ligand with a known  
31 function (such as TRAIL) to which the receptor binds,  
32 one of ordinary skill in the art cannot reasonably  
33 predict the function of the TNFR family member. [RX  
34 1049, ¶ 13.]

1           NI's own witness, Dr. Reed, did not testify that the specification and figures  
2   of the '846 application would have reasonably conveyed to one of ordinary skill in  
3   the art that a DR5 having the deduced amino acid sequence shown in Figure 1 is  
4   in fact a functional death receptor protein based solely on its amino acid  
5   sequence (primary structure). Dr. Reed did not testify that one of ordinary skill in  
6   the art would have understood the '846 application to describe a DNA encoding a  
7   functional death receptor protein. Rather, Dr. Reed testified to "the most  
8   reasonable" (not the necessary and always) conclusion that one of ordinary skill  
9   in the art would have drawn from the disclosure of the '846 application (FF 72).  
10   Dr. Reed bases this conclusion on his testimony that there was "significant"  
11   percent sequence identity between the deduced amino acid sequence of DR5's  
12   death domain and the death domains of TNFR1, Fas and DR3 (FFs 64 and 65).  
13   However, we decline to credit that testimony because Dr. Reed did not provide a  
14   sufficient basis for his opinion. Dr. Reed did not explain how percent sequence  
15   identity scores were obtained, identify what alignment methods and parameters  
16   were used by the "references" (Chinnayian and Marsters (NX 2058 and NX  
17   2059)), explain how percent identity scores based on different alignment  
18   methods and parameters relate to each other, what standard of error was  
19   typically found, whether iteration was necessary to obtain a statistically valid  
20   result, etc. 37 CFR § 41.158. Standing Order, ¶ 24. Further, as illustrated by  
21   the discussion of Hb S above, even very small differences between protein  
22   variants with highly similar amino acid sequences can produce significant  
23   differences in function.



1           Therefore, in view of the state of the art at the time the '846 application  
2           was filed and the testimony of both Drs. Reed and Cheng, we find that the '846  
3           application does not describe an isolated DNA encoding a functional protein  
4           which binds TRAIL within the scope of Count 1. Rather, the '846 application  
5           describes an isolated DNA which encodes a polypeptide which may be  
6           preliminarily classified as a TNF death receptor protein based upon its deduced  
7           amino acid sequence. However, given the unpredictability of determining  
8           function from structure, a person skilled in the art would have had to carry out  
9           further research to identify the function(s) of the protein encoded by the DNA set  
10          forth in Figure 1 of the '846 application.

11          Anticipation is a question of fact, not a conclusion of law, no matter how  
12          reasonable that conclusion may appear to be. Putative assignment to a protein  
13          (sub)family does not assess the actual biological function/utility of a gene  
14          sequence and encoded protein product given the unpredictability of determining  
15          function from structure. Ni has failed to establish that the '846 application  
16          describes a DNA encoding a functional death receptor protein based solely on  
17          the disclosure of a deduced amino acid sequence. Brenner v. Manson, 383 U.S.  
18          519, 532, 148 USPQ 689, 694 (1966) ("the presumption that adjacent  
19          homologues have the same utility has been challenged in the steroid field  
20          because of 'greater known unpredictability of compounds in that field.'").

21          Ni argues that the DR5 protein of the '846 application inherently binds  
22          TRAIL and that the '846 specification explicitly teaches that DR5 binds a TNF  
23          ligand selected from a limited list which includes TRAIL (Paper 29, p. 2, ¶ 3).

1 First, before considering whether a limitation is an inherent characteristic  
2 of an embodiment within the scope of a count, that embodiment must itself be  
3 sufficiently described and enabled. Toro, 69 USPQ2d at 1590. Thus, this  
4 argument fails because Ni has not established that the '846 application describes  
5 an enabled embodiment within the scope of Count 1 for the reasons above.

6 Second, arguing that DR5 binds a TNF ligand from a limited list which  
7 included TRAIL is also unpersuasive. The '846 specification does not explicitly  
8 identify TRAIL as the cognate ligand for DR5. The so-called "limited list" to which  
9 Ni refers apparently covers all the known and unknown ligands of the TNF family,  
10 i.e., the list enumerates the eleven then known TNF ligands and then adds a  
11 catch-all "DR5 ligands," seemingly in the event DR5 did not bind any of the then  
12 known TNF ligands. Neither the disclosure of the '846 application nor the  
13 testimony of Dr. Reed suggests that DR5 necessarily and always binds TRAIL or  
14 that DR5 binds a ligand selected from a limited subset of TNF ligands.

15 Third, while a specific DNA sequence may render a protein having a  
16 particular amino acid sequence obvious, a DNA is not a protein and, therefore,  
17 does not anticipate the encoded protein and its inherent properties.

18 Fourth, Ni's reliance on cited case law is misplaced. Ni argued that  
19 even without express appreciation of a limitation  
20 recited in a count, disclosure in a priority application  
21 of an embodiment which is later shown to *inherently*  
22 possess a characteristic satisfying that limitation is  
23 sufficient to establish constructive reduction to  
24 practice. See e.g., *Silvestri v. Grant*, 496 F.2d 593,  
25 599, 181 U.S.P.Q. 706, 710 (CCPA 1974) ("The  
26 invention is not the language of the count but the  
27 subject matter thereby defined."); See also *Hudziak v.*  
28 *Ring*, 2005 Pat. App. LEXIS 26 (Bd. Pat. App. Intf.,  
29 Sept. 2005) (confirming that a party's priority

1 applications, which disclosed an antibody but did not  
2 state the antibody bound to a particular receptor  
3 protein (HER2) as recited in the count, were  
4 nonetheless constructive reductions to practice  
5 because subsequent evidence showed that the  
6 antibody bound HER2.) [Paper 29, p. 8, ¶ 1, original  
7 emphasis.]

8 Neither Silvestri nor Hudziak are on point. Silvestri has been discussed  
9 above (§ III. Ni Substantive Motion I). In Silvestri, the court held that the  
10 evidence established that Silvestri had prepared a new form of ampicillin, that  
11 Silvestri recognized and appreciated the existence of the new form of ampicillin  
12 and that the new form of ampicillin had utility. Id., 496 F.2d at 598-601, 181  
13 USPQ at 709-712. The court acknowledged that the ampicillin of the count  
14 required a molecular weight of about 349 and greater storage stability than the  
15 previously known form of ampicillin. However, the court thought these were  
16 inherent properties of the new form of ampicillin that Silvestri was said to have  
17 obtained, recognized and described. Id., 496 F.2d at 599, 181 USPQ at 709.  
18 The court noted in Silvestri that the reduction to practice test does not require in  
19 haec verba appreciation of each of the limitations of the count:

20 This standard does not require that Silvestri establish  
21 that he recognized the invention in the same terms as  
22 those recited in the count. The invention is not the  
23 language of the count but the subject matter thereby  
24 defined. Silvestri must establish that he recognized  
25 and appreciated as a new form, a compound  
26 corresponding to the compound defined by the count.  
27 Id., 496 F.2d at 599, 181 USPQ at 710.

28 Here, the DNA of the count is a precursor to a new compound, a protein  
29 which binds TRAIL. Thus, it is necessary to consider whether the '846  
30 application describes the encoded protein and its properties/uses. While the '846

1 application describes a specific DNA, it only speculates that the DNA of Figure 1  
2 encodes a protein having the desired properties. Ni is not in the same position  
3 as Silvestri, whose application specifically described an ampicillin compound,  
4 specifically recognized it as a new form of ampicillin and specifically described  
5 certain properties of the compound. Ni's application describes a precursor to an  
6 encoded protein, but only speculates on the nature and properties of that protein.  
7 Therefore, Silvestri is not on point.

8 Similarly, in Hudziak v. Ring, 80 USPQ2d 1018, 1019 (Bd. Pat. App. & Int.  
9 2005), the count was directed to a monoclonal antibody that bound human  
10 epidermal growth factor receptor 2 (HER2). A panel of the Board decided that  
11 Chiron's (Ring's real party-in-interest) 1984 application disclosed an embodiment  
12 within the count, i.e., a murine monoclonal antibody designated 454C11. Id. The  
13 panel noted that the 1984 application (06/577,976) stated that hybridomas which  
14 produced 454C11 were deposited with the ATCC and that evidence submitted by  
15 Chiron established that 454C11 bound HER2. Id. at 1020-21.

16 83. The panel also noted in its decision (Paper 258, p. 129) that "Table 3 of  
17 the 1984 application reports the binding of antibodies to breast cancer  
18 cell lines and indicates that 454C11 binds to SKBR3 cells, which are now  
19 known to express HER2. (CX 1081, p. 3)."

20 Thus, in Hudziak, Chiron was said to have actually prepared an embodiment  
21 within the count, monoclonal antibody 454C11, and to have described it as a  
22 new protein and appreciated one of its properties/functions, i.e., that it bound to  
23 breast cancer cells. Ni's '846 application, on the other hand, describes a

1 precursor to an encoded protein, but only speculates on the nature and  
2 properties of that protein. Therefore, Hudziak is not on point.

3 Since Ni has failed to establish that '846 application describes an enabled  
4 death receptor protein of the TNF family, we do not reach the issue of what the  
5 inherent characteristics of that protein are. In both Silvestri and Hudziak, the  
6 application specifically described compounds that were recognized as novel and  
7 as having certain properties. These described and characterized compounds  
8 were later found to have other properties required by the count. Here, Ni's '846  
9 application does not describe and characterize the protein encoded by the DNA  
10 of Figure 1. Ni's application only speculates on the nature and properties of the  
11 encoded protein and that speculation is insufficient to show possession of an  
12 enabled embodiment within the count which is later found to have other  
13 properties required by the count.

14 84. Lastly, Ni argues DR5 DNA might be used as "diagnostic reagents for  
15 detecting mutated forms of DR5 associated with a dysfunction (e.g.,  
16 diseases which result from under-expression, over-expression or altered  
17 expression of DR5, such as tumors or autoimmune diseases)" or "in  
18 gene transfer applications" (Paper 29, ¶¶ bridging pp. 10-11).

19 As noted by Rauch in its opposition (Paper 53, pp. 13-14), these uses are  
20 premised on expression of the encoded DR5 protein being linked to a particular  
21 disease state or on the ability of DR5 to induce apoptosis. The '846 application  
22 does not describe how to use a DNA encoding DR5 because it only speculates  
23 on the nature and properties of DR5.

1           Based on the foregoing, Ni is not entitled to benefit of the filing date of the  
2    '846 application as to Count 1.

3           In conclusion, Ni substantive motion 2 is **granted-in-part, denied-in-part**  
4    **and dismissed-in-part.**

5    **VII.   Rauch Substantive Motion 2**

6           Pursuant to 37 CFR § 41.121(a)(1)(i), Rauch moves to redefine the scope  
7    of the interference by designating Ni claims 321, 322, 324 and 477 of the '583  
8    application as corresponding to Count 1 (Paper 34). Ni opposes (Paper 49);  
9    Rauch replies (Paper 61).

10          "A claim corresponds to a count if the subject matter of the count, treated  
11    as prior art to the claim, would have anticipated or rendered obvious the subject  
12    matter of the claim." 37 CFR § 1.207(b)(2). The subject matter of Count 1 is  
13    directed to a genus of isolated DNAs that encode a polypeptide having an amino  
14    acid sequence that is at least 90% identical to SEQ ID NO:2 of Rauch's involved  
15    '642 patent, wherein the polypeptide binds TRAIL (FF 10 and 11). It is  
16    undisputed that the TRAIL-R protein described in SEQ ID NO:2 of the '642 patent  
17    is the 440 amino acid isoform of a receptor protein alternatively referred to in the  
18    literature as TR-2, DR5, Apo-2 TRICK2 and KILLER (FF 23).

19          85. It is undisputed that the DR5 protein disclosed in the '583 application is  
20          the 411 amino acid isoform of TR-2 (see Paper 49, p. B-1, where Ni  
21          admits Rauch SMFs 6 and 7 as set forth in Paper 34, p. 10).

22          86. Amino acid residues 1 to 440 of SEQ ID NO:2 of Rauch's '642 patent are  
23          identical to amino acid residues -51 to 360 of SEQ ID NO:2 of Ni's '583

1 application except for the inclusion of additional amino acid residues 185  
2 to 213 in SEQ ID NO:2 of Rauch's '642 patent (RX 1044, pp. 58-60; RX  
3 1046, ccs. 33-35).

4 87. Amino acid residues 52-184 of SEQ ID NO:2 of Ni's 583 application  
5 constitute the extracellular domain of DR5, which is the region of DR5  
6 said to bind TRAIL (RX 1004, p. 6, ll. 6-7 and Example 6, pp. 53-54).

7 88. Claim 319 of the '583 application reads:

8 An isolated polynucleotide comprising a nucleic acid  
9 which encodes amino acids 1 to 360 of SEQ ID NO:2.

10 89. Claim 321 of the '583 application reads:

11 The polynucleotide of claim 319, wherein said nucleic  
12 acid encodes amino acids -50 to 360 of SEQ ID NO:2.

13 90. Amino acid residues 2 to 440 of SEQ ID NO:2 of Rauch's '642 patent are  
14 identical to amino acid residues -50 to 360 of SEQ ID NO:2 of Ni's '583  
15 application except for the inclusion of additional amino acid residues 185  
16 to 213 in SEQ ID NO:2 of Rauch's '642 patent (RX 1044, pp. 58-60; RX  
17 1046, ccs. 33-35).

18 91. The polynucleotide of claim 321 includes the region of DNA which  
19 encodes the extracellular domain of DR5 and, therefore, encodes a  
20 polypeptide that binds TRAIL.

21 92. Therefore, the polynucleotide of Ni claim 321 encodes a polypeptide  
22 which is at least 90% identical to the amino acid sequence of SEQ ID  
23 NO:2 of Rauch's '642 patent and which binds TRAIL.

24 93. Claim 322 of the '583 application reads:

25 The polynucleotide of claim 321, which comprises  
26 nucleotides 133 to 1362 of SEQ ID NO:1.

- 1       94. Nucleotide residues 133 to 1362 of SEQ ID NO:1 of the '583 application  
2       encode amino acid residues -50 to 360 of SEQ ID NO:2 of the '583  
3       application which are identical to amino acid residues 2 to 440 of SEQ ID  
4       NO:2 of Rauch's '642 patent except for the inclusion of additional amino  
5       acid residues 185 to 213 in SEQ ID NO:2 of Rauch's '642 patent (RX  
6       1044, pp. 58-60; RX 1046, ccs. 33-35).
- 7       95. The polynucleotide of claim 322 includes the region of DNA which  
8       encodes the extracellular domain of DR5 and, therefore, encodes a  
9       polypeptide that binds TRAIL.
- 10      96. Therefore, the polynucleotide of Ni claim 322 encodes a polypeptide  
11      which is at least 90% identical to the amino acid sequence of SEQ ID  
12      NO:2 of Rauch's '642 patent and which binds TRAIL.
- 13      97. Claim 324 of the '583 application reads:  
14             The polynucleotide of claim 322, which comprises  
15             nucleotides 130 to 1362 of SEQ ID NO:1.
- 16      98. Nucleotide residues 130 to 1362 of SEQ ID NO:1 of the '583 application  
17      encode amino acid residues -51 to 360 of SEQ ID NO:2 of the '583  
18      application which are identical to amino acid residues 1 to 440 of SEQ ID  
19      NO:2 of Rauch's '642 patent except for the inclusion of additional amino  
20      acid residues 185 to 213 in SEQ ID NO:2 of Rauch's '642 patent (RX  
21      1044, pp. 58-60; RX 1046, ccs. 33-35).
- 22      99. The polynucleotide of claim 324 includes the region of DNA which  
23      encodes the extracellular domain of DR5 and, therefore, encodes a  
24      polypeptide that binds TRAIL.



- 1           100.       Therefore, the polynucleotide of Ni claim 324 encodes a  
2                    polypeptide which is at least 90% identical to the amino acid sequence of  
3                    SEQ ID NO:2 of Rauch's '642 patent and which binds TRAIL.
- 4           101.       Claim 476 of the '583 application reads:  
5                    An isolated polynucleotide comprising a nucleic acid  
6                    which encodes the mature amino acid sequence  
7                    encoded by the cDNA clone in ATCC Deposit No.  
8                    97920.
- 9           102.       Claim 477 of the '583 application reads:  
10                   The polynucleotide of claim 476, wherein said nucleic  
11                   acid encodes the complete amino acid sequence  
12                   encoded by the cDNA clone in ATCC Deposit No.  
13                   97920.
- 14          103.       According to the '583 specification, the cDNA in ATCC Deposit No.  
15                   97920 encodes a DR5 polypeptide having the amino acid sequence set  
16                   forth in SEQ ID NO:2 (RX 1044, p. 9, ll. 5-8).
- 17          104.       The polynucleotide of claim 477 includes the region of DNA which  
18                   encodes the extracellular domain of DR5 and, therefore, encodes a  
19                   polypeptide which binds TRAIL.
- 20          105.       Therefore, the polynucleotide of Ni claim 477 encodes a  
21                   polypeptide which is at least 90% identical to the amino acid sequence of  
22                   SEQ ID NO:2 of Rauch's '642 patent and which binds TRAIL.
- 23          106.       In essence, Rauch's position is that "as long as a single species of  
24                   a claim falls within the count, then that claim corresponds to the count"  
25                   (Paper 34, p. 5, ¶ 1).
- 26          Rauch has established that each species of isolated polynucleotide recited  
27          in Ni claims 321, 322, 324 and 477 falls within the generic isolated TRAIL-R DNA

1 of Count 1 (FF 73-92). Rauch's position is that "if Count 1 were prior art to the Ni  
2 claims, it would anticipate the claims" (Paper 34, p. 5, ¶ 2).

3 A prior art species within a claimed genus reads on the generic claim and  
4 anticipates. In re Gostelli, 872 F.2d 1008, 1010, 10 USPQ2d 1614, 1616 (Fed.  
5 Cir. 1989). However, a species claim is not necessarily obvious in light of a prior  
6 art disclosure of a genus. In re Baird, 16 F.3d 380, 382, 29 USPQ2d 1550, 1552  
7 (Fed. Cir. 1994). In other words, the "earlier disclosure of a genus does not  
8 necessarily prevent patenting a species member of that genus." Eli Lilly & Co. v.  
9 Bd. of Regents of the Univ. of Washington, 334 F.3d 1264, 1270, 67 USPQ2d  
10 1161, 1165 (Fed. Cir. 2003)(citing Bristol-Myers Squibb Co. v. Ben Venue Labs.,  
11 Inc., 246 F.3d 1368, 1380, 58 USPQ2d 1508, 1516-17(Fed. Cir. 2001)).

12 Here, Rauch has the burden of establishing that each of Ni claims 321,  
13 322, 324 and 477 would have been anticipated or rendered obvious by the  
14 subject matter of Count 1. 37 CFR § 41.121(b). Simply showing that a species  
15 claim falls within the subject matter of a generic count (see Paper 34, pp. 5-7)  
16 does not suffice to establish that the claim is anticipated or rendered obvious by  
17 the subject matter of the count. Rauch has not established why any of Ni claims  
18 321, 322, 324 and 477 would be unpatentable over the subject matter of Count 1,  
19 i.e., why each of these claims is an obvious species within the generic subject  
20 matter of the count. Therefore, Rauch has failed to meet its burden.

21 Based on the foregoing, Rauch substantive motion 2 is **denied**.

22

23

1 **VIII. Rauch Substantive Motion 3**

2 Pursuant to 37 CFR § 41.121(a)(1)(iii) and the Order issued 29 November  
3 2005 (Paper 26), Rauch moves for judgment that Ni claims 287, 289-299, 351-  
4 361, 389-403, 431, 432, 434-442, 446, 448-458, 507-517, 581 and 623-632 ("Ni  
5 claims at issue") are unpatentable under 35 U.S.C. §§ 102(a) and/or (e) as  
6 clearly anticipated by one or more of U.S. Patent 6,642,358 ("the '358 patent,"  
7 RX 1042), U.S. Patent 6,072,047 ("the '047 patent," RX 1048), U.S. Patent  
8 6,569,642 ("the '642 patent," RX 1046) and WO 98/35986 ("WO '986," RX 1032)  
9 (collectively, "the Rauch references") (Paper 35, ¶¶ bridging pp. 2-3). Ni opposes  
10 (Paper 50); Rauch replies (Paper 63).

11 107. The '358 patent issued 4 November 2003, based on application  
12 09/578,392, filed 25 May 2000, which is a divisional of application  
13 08/883,036, filed 26 June 1997, which is a continuation-in-part of  
14 application 08/869,852, filed 4 June 1997, which is a continuation-in-part  
15 of application 08/829,536, filed 28 March 1997, which is a continuation-  
16 in-part of application 08/815,255, filed 12 March 1997, which is a  
17 continuation-in-part of application 08/799,861, filed 13 February 1997  
18 (RX 1042, title page).

19 108. The '047 patent issued 6 June 2000 based on application  
20 08/883,036, filed 26 June 1997, which is a continuation-in-part of  
21 application 08/869,852, filed 4 June 1997, which is a continuation-in-part  
22 of application 08/829,536, filed 28 March 1997, which is a continuation-  
23 in-part of application 08/815,255, filed 12 March 1997, which is a

1 continuation-in-part of application 08/799,861, filed 13 February 1997  
2 (RX 1048, title page).

3 109. The '642 patent issued 27 May 2003, based on application  
4 09/536,201, filed 27 March 2000, which is a continuation of application  
5 08/883,036, filed 26 June 1997, which is a continuation-in-part of  
6 application 08/869,852, filed 4 June 1997, which is a continuation-in-part  
7 of application 08/829,536, filed 28 March 1997, which is a continuation-  
8 in-part of application 08/815,255, filed 12 March 1997, which is a  
9 continuation-in-part of application 08/799,861, filed 13 February 1997  
10 (RX 1046, title page).

11 110. WO '986 published 20 August 1998, based on international  
12 application PCT/US98/02239, filed 11 February 1998 (RX 1032, title  
13 page).

14 According to the relevant paragraphs of 35 U.S.C. § 102:

15 A person shall be entitled to a patent unless--

16 (a) the invention was known or used by others in this  
17 country, or patented or described in a printed  
18 publication in this or a foreign country before the  
19 invention thereof by the applicant for patent, or

20 \* \* \* \* \*

21 (e) the invention was described in (1) an application  
22 for a patent, published under section 122(b), by  
23 another filed in the United States before the invention  
24 by the applicant for patent or (2) a patent granted on  
25 an application for patent by another filed in the United  
26 States before the invention by the applicant for patent,  
27 except that an international application filed under the  
28 treaty defined in section 351(a) shall have the effects  
29 for the purposes of this subsection of an application  
30 filed in the United States only if the international

1 application designated the United States and was  
2 published under Article 21(2) of such treaty in the  
3 English language, or

4 \* \* \* \* \*

5 References based on international applications that were filed prior to 29  
6 November 2000 are subject to the former version of 35 U.S.C. § 102(e),<sup>8</sup> i.e.,

7 A person shall be entitled to a patent unless --

8 (e) the invention was described in a patent granted on  
9 an application for patent by another filed in the United  
10 States before the invention thereof by the applicant  
11 for patent, or on an international application by  
12 another who has fulfilled the requirements of  
13 paragraphs (1), (2), and (4) of section 371(c) of this  
14 title before the invention thereof by the applicant for  
15 patent.

16 A prima facie case is made out under § 102(a) if, within a year of the filing  
17 date, the invention, or an obvious variant thereof, is described in a "printed  
18 publication" whose authorship differs from the inventive entity unless it is stated  
19 within the publication itself that the publication is describing the applicant's work.  
20 In re Katz, 687 F.2d 450, 215 USPQ 14 (CCPA 1982).

21 111. None of the Rauch references issued or published prior to the 17  
22 March 1998 filing date of Ni's claims at issue.<sup>9</sup>

23 112. None of the Rauch references qualify as prior art under § 102(a)  
24 vis-à-vis Ni's claims at issue.

25 Therefore, to the extent Rauch substantive motion 3 seeks a judgment that  
26 any of Ni claims at issue are unpatentable under § 102(a) over any of the Rauch

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<sup>8</sup> Pursuant to § 13205 of Pub. L. 107-273.

<sup>9</sup> Rauch has not argued prior public knowledge or use of the subject matter of any of Ni's claims at issue.

1 references, the motion is **denied**. We now consider whether any of the Rauch  
2 references qualify as prior art under § 102(e).

3 WO '986 is based on an international application filed prior to 29 November  
4 2000 (FF 110). Therefore, it must satisfy the requirements of then applicable  
5 former § 102(e) in order to qualify as prior art. Rauch has neither argued nor  
6 shown that WO '986 satisfies the requirements of the applicable § 102(e) (see  
7 Paper 35, p. 19, ¶ 1). Thus, Rauch has not established that WO '986 qualifies as  
8 prior art under the applicable § 102(e) vis-à-vis Ni's claims at issue.

9 Consequently, to the extent Rauch substantive motion 3 seeks a judgment that  
10 any of Ni claims at issue are unpatentable under § 102(e) as anticipated by WO  
11 '986, the motion is **denied**.

12 As indicated above (FF 107-109), the '358, '047 and '642 patents are  
13 related. The '047 patent issued based on application 08/833,036 and the '358  
14 and '642 patents issued based on an application identified as a divisional or a  
15 continuation, respectively, of application 08/883,036, filed on 26 June 1997. The  
16 filing date of the 08/833,036 application is prior to the 17 March 1998 filing date  
17 of Ni's involved claims and prima facie qualifies as prior art under § 102(e)  
18 against Ni's claims at issue. It is not necessary to consider whether Ni's claims  
19 at issue are anticipated by the '358 and '642 patents, if Ni's claims are anticipated  
20 by the '047 patent.

21 Claim chart appendix G attached to Rauch substantive motion 3 correlates  
22 the disclosure of the '047 patent to each of the limitations of each of Ni's claims  
23 at issue. Therefore, Rauch substantive motion 3, when considered in light of the

1 evidence relied upon in support of the motion, establishes a sufficient basis for  
2 holding Ni's claims at issue prima facie unpatentable under 35 U.S.C. § 102(e) as  
3 anticipated by the '047 patent.

4 113. Ni does not contest that the '047 patent describes the subject  
5 matter of its claims at issue.

6 114. Rather, Ni contends that the '047 patent does not qualify as prior art  
7 because Ni's '583 application claims are argued to be entitled to benefit  
8 of the 17 March 1997 filing date of its '846 provisional application (Paper  
9 50, pp. 3-4; p. 10, ¶ 1; and Appendix E).<sup>10</sup>

10 115. Rauch maintains that Ni cannot obtain benefit of its '846 application  
11 due to a lack of utility (Paper 35, p. 2, ¶ 3 and p. 19, ¶ 2 - p. 21, ¶ 1).

12 As stated in In re Fisher, 421 F.3d 1365, 1378 USPQ2d 1225, 1235 (Fed.  
13 Cir. 2005),

14 [i]t is well established that the enablement  
15 requirement of § 112 incorporates the utility  
16 requirement of § 101. The how to use prong of  
17 section 112 incorporates as a matter of law the  
18 requirement of 35 U.S.C. § 101 that the specification  
19 disclose as a matter of fact a practical utility for the  
20 invention. If the application fails as a matter of fact to  
21 satisfy 35 U.S.C. § 101, then the application also fails  
22 as a matter of law to enable one of ordinary skill in the  
23 art to use the invention under 35 U.S.C. § 112.

24 The salient question is whether Ni's claims at issue are entitled to benefit of  
25 the 17 March 1997 filing date of Ni's '846 provisional application, thereby  
26 antedating the 26 June 1997 filing date of the '047 patent. Benefit for purposes

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<sup>10</sup> We need not consider whether Ni's '583 application claims are entitled to § 119(e) benefit of the 29 July 1997 filing date of Ni's '021 provisional application because the 29 July 1997 is after the 26 June 1997 filing date of the 08/833,036 application which issued as Rauch's '047 patent.

1 of antedating prior art, in this case, benefit under 35 U.S.C. § 119(e), is different  
2 from benefit for the purpose of priority. As set forth in section 119(e) of Title 35  
3 of the United States Code,

4 [a]n application for a patent filed under section 111(a)  
5 or section 363 of this title for an invention disclosed in  
6 the manner provided by the first paragraph of section  
7 112 of this title in a provisional application filed under  
8 section 111(b) of this title, by an inventor or inventors  
9 named in the provisional application, shall have the  
10 same effect, as to such invention, as though filed on  
11 the date of the provisional application filed under  
12 section 111(b) of this title, if the application for patent  
13 filed under section 111(a) or section 363 of this title is  
14 filed not later than 12 months after the date on which  
15 the provisional application was filed and if contains or  
16 is amended to contain a specific reference to the  
17 provisional application.

18 116. Ni's '583 application claims benefit under § 119(e) of its '846  
19 provisional application filed 17 March 1997 (RX 1044, p. 83).

20 The parties disagree whether the disclosure of Ni's '846 application satisfies the  
21 description and enablement requirements of § 112, first paragraph, as to the full  
22 scope of the subject matter of Ni's '583 application claims at issue.

23 Ni cites to specific disclosures in its '846 application said to describe every  
24 element of its claims at issue (Appendix E attached to Ni opposition 3 to Rauch  
25 motion 3). Ni argues that the '846 application discloses that DR5 DNA can be  
26 used to express (produce) DR5 polypeptides, which in turn, can be used (a) to  
27 make anti-DR5 antibodies for treating diseases associated with apoptosis or (b)  
28 as diagnostic reagents for detecting mutated forms of DR5 associated with a  
29 dysfunction or (c) as antagonists of DR signaling (Paper 50, pp. 10-13). Ni  
30 further argues that the DR5 DNA molecule itself can be used (d) as diagnostic



1 reagents to detect mutated forms of DR5 associated with a dysfunction, e.g.,  
2 diseases which result from under-expression, over-expression or altered  
3 expression of DR5 or (e) in gene transfer applications to increase cellular  
4 apoptosis (Paper 50, pp. 13-14). Ni still further argues that DR5 antisense DNA  
5 or RNA can be used to inhibit DR5 gene expression (Paper 50, p. 14).

6 117. Dr. Reed, testifying for Ni, stated that the technology necessary to  
7 achieve these functions was within routine skill in the art, e.g., a skilled  
8 artisan would know how to express and purify a protein (e.g., DR5) from  
9 cDNA (e.g., DR5 cDNA), how to produce antibodies that bind a desired  
10 protein (e.g., DR5), etc. (e.g., NX 2099, ¶ 35-46).

11 118. Dr. Reed further testified that the uses for DR5 DNA and its  
12 encoded DR5 polypeptide described in the '846 application would have  
13 been believable to one of ordinary skill in the art because the asserted  
14 uses had previously been shown to be recognized uses of TNF death  
15 receptors TNFR1, Fas and/or DR3 (NX 2099, 33-34, 47-52).

16 Essentially, Dr. Reed's testimony as to the utility/enablement of DR5 DNA or  
17 its encoded DNA polypeptide is based on the assumption that the DR5 DNA  
18 described in the '846 application encoded a functional TNF death receptor and,  
19 therefore, what was known about the use of other death receptors was applicable  
20 to DR5 DNA and its encoded protein (see e.g., NX 2099, ¶¶ 49 and 50 ("[b]ased  
21 on precedent from prior work in the field of TNF-family receptors" and "[b]ased on  
22 precedent from the literature where agonistic and antagonistic antibodies to other  
23 TNF-family death receptors had been produced and characterized,"

1 respectively)). According to Ni, Dr. Reed "has testified unequivocally that 'you  
2 can reasonably make a prediction based on homology alone" and by analyzing  
3 "the particular subfamily of proteins to which DR5 belongs, *i.e.*, death receptors",  
4 "the most reasonable conclusion to draw from Ni's March 17, 1997 application is  
5 that DR5 is expected, by persons of ordinary skill in the art to be a novel death  
6 receptor [and that] a person of ordinary skill in the art would have predicted that  
7 activation of DR5 would induce apoptosis" (Paper 50, p. 16, ¶ 2, citations  
8 omitted). The disclosure cited by Ni in its Appendix E is no more specific than  
9 Dr. Reed's testimony. For example, in the second paragraph of the third column  
10 on page 2 of Appendix E, Ni points to page 6, lines 28-32 of the '846 application  
11 as disclosing that "[t]he homology DR5 shows to other death domain containing  
12 receptors strongly indicates that DR5 is also a death domain containing receptor  
13 with the ability to induce apoptosis." According to Ni, Dr. Reed properly focused  
14 on the subset of known death receptors and the "single" function that unites  
15 them, *i.e.*, their ability to induce apoptosis (Paper 50, pp. 15-16).

16 Rauch, on the other hand, argues that sequence homology alone is  
17 insufficient to establish that the encoded DR5 polypeptide disclosed in the '846  
18 application is in fact a TNF family death domain receptor. According to Rauch,  
19 unless the disclosure of the '846 application shows DR5 to be an actual TNF  
20 family member receptor, *e.g.*, by identification of a known TNF ligand as its  
21 cognate ligand or by specific experimental data showing that DR5 induces a  
22 TNFR-mediated biological activity, *e.g.*, apoptosis, inflammatory response, etc.,

1 the '846 application fails to disclose a specific, substantial and credible utility for  
2 the encoded protein and, therefore, for the claims at issue.

3 119. Dr. Cheng, testifying for Rauch, stated that Ni's '846 application  
4 discloses the DNA and amino acid sequence of the  
5 411 amino acid isoform of TR-2, which they refer to  
6 as DR5. DR5 was identified based on sequence  
7 homology to other death domain-containing members  
8 of the TNFR family, including TNFR-1, DR3, and Fas  
9 ('846 Provisional, page 5, lines 21-24). The  
10 applicants assert that agonists to DR5 can be used to  
11 increase apoptosis, while antagonists to DR5 can be  
12 used to inhibit apoptosis. This assertion is based  
13 entirely on sequence homology between DR5 and the  
14 death domain-containing receptors TNFR-1, DR3,  
15 and Fas. However, the '846 Provisional does not  
16 identify a ligand for DR5 and does not contain any  
17 experimental data regarding DR5 function.

18 Sequence homology to other death domain-  
19 containing receptors may be sufficient to convince  
20 one of ordinary skill in the art that a novel protein is a  
21 TNFR family member. However, sequence homology  
22 alone is not sufficient to support an assertion that a  
23 novel TNFR family member will induce specific  
24 biological activities such as apoptosis. Without  
25 additional data regarding the activity of a TNFR family  
26 member, such as, for example, the identity of the  
27 ligand with a known function (such as TRAIL) to which  
28 the receptor binds, one of ordinary skill in the art  
29 cannot reasonably predict the function of the TNFR  
30 family member. This is because TNFR family  
31 members are involved in complex signal transduction  
32 pathways, which can affect a wide spectrum of  
33 biological activities including apoptosis, inflammatory  
34 response, cell proliferation, cell survival, and other  
35 activities. The binding of certain TNFR family  
36 members by their corresponding ligands can lead to  
37 activation of multiple signal transduction pathways. As  
38 stated above, Ni's '846 Provisional contains no data  
39 regarding the ligand for DR5, nor does it disclose  
40 experimental data of its function. Without knowing  
41 more information about the activity of DR5, such as  
42 for example its specificity for a ligand with a known  
43 function, one of ordinary skill in the art could not

1 reasonably predict the function of the TNFR family  
2 member. [RX 1049, ¶¶ 12-13.]

3 For essentially the reasons set forth in our analysis in "§ VI. Ni Substantive  
4 Motion 2" above, we credit the testimony of Dr. Cheng over that of Dr. Reed. In  
5 short, one of ordinary skill in the art might classify the product encoded by the  
6 DNA set forth in Figure 1 of the '846 application as a possible TNF death  
7 receptor protein based on the deduced amino acid sequence of the product.  
8 However, given the unpredictability of determining function from structure (the  
9 "Holy Grail" of molecular biology), a skilled artisan would have had to carry out  
10 further research to identify the function(s) of the protein encoded by the DNA set  
11 forth in Figure 1 of the '846 application. Thus, the disclosure of the '846  
12 application fails to satisfy the "how-to-use" requirement of § 112, first paragraph,  
13 as to the subject matter of the Ni's claims at issue. Ni's claims at issue are not  
14 entitled to § 119(e) benefit of the filing date of the '846 application and the '047  
15 patent still qualifies as prior art under § 102(e). Therefore, Ni claims 287, 289-  
16 299, 351-361, 389-403, 431, 432, 434-442, 446, 448-458, 507-517, 581 and 623-  
17 632 ("Ni's claims at issue") are unpatentable under 35 U.S.C. § 102(e) as clearly  
18 anticipated by U.S. Patent 6,072,047. It is not necessary to consider whether  
19 Ni's claims at issue are also anticipated by either patent '358 or '642.

20 In its opposition, Ni argues that Rauch substantive motion 3 should be  
21 denied on procedural grounds because it does not seek judgment that all of Ni's  
22 corresponding claims are unpatentable and, therefore, is not a proper threshold  
23 motion (Paper 50, p. 2, ¶ 3 - p. 2, ¶ 2; p. 7, ¶ 2 - p. 9, ¶ 3). Rauch substantive  
24 motion 3 is an ordinary attack on patentability. Ni has not provided any basis

1 requiring a motion for unpatentability to attack all of a party's involved claims and  
2 we know of none. Therefore, this argument is without merit.

3 Based on the foregoing, Rauch substantive motion 3 is **granted to the**  
4 **extent** that Ni's claims 287, 289-299, 351-361, 389-403, 431, 432, 434-442, 446,  
5 448-458, 507-517, 581 and 623-632 are unpatentable under 35 U.S.C. § 102(e)  
6 as clearly anticipated by U.S. Patent 6,072,047 and **otherwise denied**.

7 **IX. Rauch Miscellaneous Motion 5**

8 Pursuant to 37 CFR § 41.115(c), Rauch seeks to exclude selected  
9 portions of the direct testimony of Dr. Reed that reference a person of ordinary  
10 skill in the art from evidence (NX 2099, ¶¶ 16, 19, 21-28, 30-43, 45-48, 50-52, 56,  
11 63 and 64), contending that his definition of ordinary skill "is so broad that it fails  
12 to limit 'one of ordinary skill in the art' to any substantive or realistic meaning of  
13 such person" (Paper 71, ¶ bridging pp. 4-5). Rauch further seeks to exclude  
14 selected portions of the redirect testimony of Dr. Reed from evidence as leading  
15 and prejudicial (NX 2123, p. 172, l. 20; p. 173, ll. 7-8; p. 172, l. 25 through p. 173,  
16 l. 2) (*id.*, p. 8, ¶¶ 2-3). Ni opposes (Paper 75); Rauch replies (Paper 83).

17 120. Rauch timely filed its objections to evidence sought to be excluded  
18 (RX 1093 and NX 2123, p. 172, l. 20 and p. 173, ll. 7-8).

19 Rauch identifies the objected to testimony of Dr. Reed as submitted in  
20 support of Ni substantive motion 2, Ni reply 2 and Ni opposition 3 to Rauch  
21 substantive motion 3 (Paper 71, Appendix D). First, Rauch's arguments go to the  
22 weight to be accorded Dr. Reed's testimony, not to its admissibility. Second,  
23 having considered the testimony of both Dr. Reed and Dr. Cheng, we credited

1 the testimony of the latter over that of the former as discussed in our denial of the  
2 relevant portion of Ni substantive motion 2 and in our granting of the relevant  
3 portion of Rauch substantive motion 3. Therefore, Rauch substantive motion 5 is  
4 **dismissed** as moot since we have not relied upon either the direct or redirect  
5 testimony of Dr. Reed to Rauch's detriment.

6 **X. Ni Miscellaneous Motion 4**

7 Pursuant to 37 CFR § 41.155(c), Ni seeks to exclude from evidence:

8 (a) exhibits related to Rauch's priority statements in (i) related interference  
9 105,240 (RX 1025 and RX 1038), (ii) this interference (RX 1051) and (iii) related  
10 interference 105,381 (RX 1052 and RX 1054);

11 (b) direct (RX 1074) and deposition testimony (NX 2179-2181) of Dr.

12 Gavin R. Scranton in related interference 105,240;

13 (c) direct testimony of Norman Boiani (RX 1075); and

14 (d) selected portions of the deposition testimony of Dr. Cheng (NX 2124,  
15 p. 132, l. 16 through p. 135, l. 5; p. 135, l. 9 through p. 136, l. 13 (with errata  
16 sheets)) (Paper 80). Rauch opposes (Paper 76); Ni replies (Paper 82).

17 Ni contends (Paper 80, pp. 22-23) that

18 RX 1025, RX 1038, RS 1051, RX 1052 and RX 1054  
19 should be excluded under FRE 901 for lack of  
20 authentication and lack of foundation. In addition,  
21 these exhibits should be excluded under FRE 1001  
22 (4), 1002, and 1003, *inter alia*, because none of these  
23 exhibits appear to be originals nor admissible  
24 duplicates of the originals. Furthermore, these  
25 exhibits should be excluded under FRE 403, *inter alia*,  
26 because its probative value, if any, is outweighed by  
27 considerations of waste of time, lack of authentication  
28 and the reliability of the copies.

1 Furthermore, RX 1074, the declaration of Dr.  
2 Gavin R. Sreaton, should be excluded under FRE  
3 403 because its probative value, if any, is outweighed  
4 by confusion of the issues. In addition, RX 1074  
5 should be excluded under 37 C.F.R. § 41.122(b)  
6 because the declaration does not respond to  
7 arguments raised in an opposition but merely is an  
8 attempt by Rauch to make additional arguments in a  
9 reply that should have been raised in a motions.  
10 Furthermore, contingent upon the Board excluding RX  
11 1074, Party Ni moves to exclude NX 2179, NX 2080  
12 and NX 2181 for being irrelevant under FRE 401 and  
13 confusing the issues under FRE 403.

14 In addition, Party Ni moves to exclude RX  
15 1075, the Declaration of Norman Boiani under FRE  
16 1002 because Exhibit A appears to be a photocopy,  
17 not an original, of a laboratory notebook page.  
18 Furthermore, Party Ni moves to exclude RX 1075  
19 under FRE 403 because Exhibit A is taken out of  
20 context of the rest of the laboratory notebook. Party  
21 Ni's inability to determine the context of Exhibit A is  
22 unfairly prejudicial and this prejudice far outweighs  
23 any probative value of RX 1075.

24 Lastly, the above-cited portions of NX 2124  
25 should be excluded under FRE 611(c), FRE 403, and  
26 Cross Examination Guideline [3] of the Standing  
27 Order. The leading questions asked by Rauch's  
28 counsel clearly suggested single answers to the  
29 witness which resulted in the interjection of the  
30 opinions of counsel for Rauch in place of Dr. Cheng's  
31 opinions. Thus, the prejudicial effect of the cited  
32 testimony far outweighs its probative value, and the  
33 above-cited evidence should be excluded or, at most,  
34 accorded little weight by the Board.

35 Ni's motion has serious procedural defects. Rule 155(c) provides that a  
36 motion to exclude evidence must explain the objections and identify the  
37 objections in the record in order. As explained in Standing Order ¶ 21.3(a) a  
38 motion to exclude evidence shall (1) identify where in the record the objection  
39 was originally made and (2) identify where in the record the evidence was relied

1 upon by the opponent, and (3) address objections to exhibits (in whole or in part)  
2 in exhibit numerical order. According to Standing Order ¶ 21.1, the objection to  
3 the admissibility of evidence should be filed as part of a motion to exclude the  
4 evidence.

5 121. Ni contends that it timely objected to exhibits RX 1025, RX 1038,  
6 RX 1051, RX 1052 and RX 1054 as shown in exhibits NX 2192 and NX  
7 2193, filed in support of its motion.

8 122. Ni exhibits NX 2192 and NX 2193 are "REDACTED" papers entitled  
9 "NI OBJECTIONS TO THE ADMISSIBILITY OF RAUCH'S  
10 SUPPLEMENTAL EXHIBIT 1054 AND RAUCH'S RESPONSES TO NI'S  
11 OBJECTIONS TO EXHIBITS 1050-1052" and "NI OBJECTIONS TO THE  
12 ADMISSIBILITY OF RAUCH EXHIBITS 1050, 1051 and 1052,"  
13 respectively.

14 123. Ni has not provided evidence that it timely objected to exhibits RX  
15 1025 and RX 1038.

16 124. Ni has not identified where in the record exhibits RX 1025, RX  
17 1038, RX 1051, RX 1052, RX 1074 and RX 1075 were relied upon by its  
18 opponent Rauch.

19 125. Rauch's exhibit list filed when it submitted its record for decision on  
20 motion ("Rauch's exhibit list," Paper 87, p. 7) identifies exhibits RX 1052  
21 and 1054 as documents upon which Rauch will rely to prove its earliest  
22 corroborated conception of the subject matter of the count in related  
23 interference 105,381.



1           126.       Similarly, Rauch's exhibit list identifies exhibit RX 1074 as the  
2           declaration of Dr. Gavin R. Scranton filed in related interference 105,240  
3           (Paper 87, p. 10).

4           127.       Moreover, a cursory review of Rauch's exhibit list clearly indicates  
5           that exhibits RX 1025, RX 1038 and RX 1075 are not of record in this  
6           interference and, therefore, are not at issue in this interference (Paper  
7           87, pp. 4-5).

8           128.       Furthermore, according to Ni, "even though Party Rauch does not  
9           appear to have yet relied on any of Rauch Exhibits   **1051** and **1052** in  
10          support of a motion. Party Ni serves Party Rauch with these objections  
11          to provide notice to Party Rauch that if, and when, any of Rauch Exhibits  
12                   **1051** and **1052** are relied upon, the following objections will be  
13          raised, unless cured by Party Rauch" (NX 2193, p. 1, ¶ 1).

14          Thus, Ni has failed to object timely to evidence it seeks to exclude (RX 1025  
15          and RX 1038). Furthermore, Ni is seeking to exclude evidence which is either  
16          not of record in this interference (RX 1025, RX 1038, RX 1074 and RX 1075)  
17          and/or has not been relied upon by Rauch in this interference (RX 1025, RX  
18          1038, RX 1052, RX 1054, RX 1074 and RX 1075). Therefore, Ni miscellaneous  
19          motion 4 to exclude evidence is **denied** as to exhibits RX 1025, RX 1038, RX  
20          1052, RX 1054, RX 1074, RX 1075 and NX 2179-2181.

21          129.       Exhibit 1051 is identified in Rauch's exhibit list as a document said  
22          to prove Rauch's earliest corroborated conception of the invention of the  
23          count in this interference (Paper 87, p. 7).

1 According to 37 CFR § 41.204(a)(2)(iv), a party filing a priority statement  
2 must "[p]rovide a copy of the earliest document upon which the party will rely to  
3 show conception." Exhibit 1051 was submitted by Rauch in fulfillment of that  
4 requirement (FF 120). Ni does not contend that Rauch has relied on exhibit RX  
5 1051 in support of any of Rauch's motion papers. The time for Rauch to lay a  
6 foundation for and authenticate its exhibit RX 1051 is when Rauch relies upon  
7 the exhibit, i.e., as part of its priority motion. The time for us to weigh the  
8 reliability and probative value of exhibit RX 1051 is when it is submitted into  
9 evidence as party of Rauch's priority motion when the motion is filed. Therefore,  
10 Ni miscellaneous motion 4 to exclude evidence is **denied** as to exhibit RX 1051.

11 130. As to the last evidence as issue, selected portions of the deposition  
12 testimony of Dr. Cheng (NX 2124, p. 132, l. 16 through p. 135, l. 5; p.  
13 135, l. 9 through p. 136, l. 13 (with errata sheets)), Ni argues that this  
14 evidence was relied upon in Rauch reply 1 and Rauch reply 4 (Paper 80,  
15 p. B2, SMF 10).

16 131. Ni explicitly directs our attention (Paper 80, pp. 17-18) to the  
17 following testimony as an example of how the direct examination of Dr.  
18 Cheng violates FRE 611(c), FRE 403 and Cross Examination Guideline  
19 [3]:

20 MR. WISE: Okay. Back on the record.

21 Q. I want to have you focus on paragraph 10.  
22 Paragraph 10 you said, "The specification of the '861  
23 application also contains additional substantial  
24 disclosure regarding antibodies to TRAIL-R, including  
25 methods for obtaining these antibodies and methods

1 of obtaining antigen binding fragments of these  
2 antibodies."

3 And it says "'861 application, page 13, line 14 to page  
4 15, line 6."

5 Where in the specification of the '861 application  
6 would you find additional substantial disclosure  
7 relating to the antibodies for TRAIL-R?

8 A. You mean where I can find the information?

9 Q. Yes.

10 A. That's indicated here is the page 13 and the line  
11 14 to 15, line 14 through page 15 of line 6.

12 Q. Okay. Can you direct me to that, please.

13 A. Where is the --

14 Q. You have that there. You were looking at the  
15 claims and you were going to show me support and  
16 specification.

17 MR. GOLDSTEIN: Objection.

18 THE WITNESS: So it's indeed in the page is 13,  
19 there is a title, "Antibodies" section, and talking about  
20 how antibody generated, including the monoclonal  
21 and polyclone antibodies.

22 MR. GOLDSTEIN: I am going to move to strike the  
23 question and the answer.

24 First, since Rauch responsive motion 4 was dismissed as moot, we did not  
25 reach Rauch reply 4. Second, Ni did not explain where and how Rauch relied  
26 upon the objected to portions of Dr. Cheng's testimony in Rauch reply 1 to  
27 support its position, e.g., how does Rauch rely upon this allegedly elicited  
28 testimony to support its motion 1 for benefit of the filing date of an earlier  
29 application for the subject matter of a count directed to isolated nucleotides.  
30 Third, to the extent Ni argues the objected portions of Dr. Cheng's testimony are

1 irrelevant, confusing or prejudicial, that objection goes to the weight to be  
2 accorded the testimony. We have accorded Dr. Cheng's testimony the weight  
3 appropriate to its relevance and the underlying facts and data relied upon in  
4 support of his opinion. Ni has not shown otherwise. Therefore, Ni miscellaneous  
5 motion 4 to exclude evidence is **denied** as to selected portions of the deposition  
6 testimony of Dr. Cheng (NX 2124, p. 132, l. 16 through p. 135, l. 5; p. 135, l. 9  
7 through p. 136, l. 13 (with errata sheets)).

8 Therefore, Ni miscellaneous motion 4 is **denied**.

9 **XI. Order**

10 Based on the foregoing and for the reasons given, it is

11 ORDERED that Ni substantive motion 1 is **denied**;

12 FURTHER ORDERED that Rauch responsive motion 4 is **dismissed** as  
13 moot;

14 FURTHER ORDERED that Rauch substantive motion 1 is **granted**;

15 FURTHER ORDERED that Ni substantive motion 2 is **granted-in-part**,  
16 **denied-in-part** and **dismissed-in-part**;

17 FURTHER ORDERED that Rauch substantive motion 2 is **denied**;

18 FURTHER ORDERED that Rauch substantive motion 3 is **granted-in-**  
19 **part** to the extent Ni claims 287, 289-299, 351-361, 389-403, 431, 432, 434-442,  
20 446, 448-458, 507-517, 581 and 623-632 are unpatentable under 35 U.S.C. §  
21 102(e) as clearly anticipated by U.S. Patent 6,072,047;

22

- 1           FURTHER ORDERED that Rauch miscellaneous motion 5 is **dismissed**  
2   as moot; and,  
3           FURTHER ORDERED that Ni miscellaneous motion 4 is **denied**.

/Richard E. Schafer/ )  
RICHARD E. SCHAFER )  
Administrative Patent Judge )

/Adriene L. Hanlon/ )  
ADRIENE LEPIANE HANLON )  
Administrative Patent Judge )

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# Molecular Biology and Biotechnology

## A Comprehensive Desk Reference

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Sequences of nucleic acids in DNA and RNA and of amino acids in proteins define the primary structure of these molecules. Sequence analysis is carried out using computer programs that implement algorithms to determine sequence properties and to compare sequences. Sequence comparison can indicate whether an RNA or protein molecule or region of DNA is already known (identity) or has some degree of similarity to a known sequence. Sequence similarity may indicate similar structure or function. Sequence analysis can suggest the function of an unknown sequence based on the features it contains. Sequence analysis is a necessary preliminary to detailed experimental studies of structure, function, and interactions of biological macromolecules. Sequences are the information repository of the cell and a natural index to our growing understanding of cellular processes as dynamic systems of interactions between macromolecules.

## 1 PURPOSE OF SEQUENCE ANALYSIS

### 1.1 PREDICTION OF FUNCTION

Sequences that are unlike any known sequence may still be made to yield information that can suggest their possible function. The function of nucleic acids and proteins depends on their structure and involves complex interactions in three dimensions. It is not presently understood whether it is possible, in general, to derive structure from sequence. Sequence alone is therefore often inadequate to determine function. Predictions made from sequence analysis need to be experimentally tested. Nevertheless, computer analysis of sequences is valuable in suggesting the most useful experiments to perform.

### 1.2 REVEALING SIMILARITY

The first thing to do with a newly determined sequence is to compare it with all known sequences. The outcome may show identity to a known sequence, which may prove disappointing if one is hoping for something new. Similarity to a known sequence may suggest something new that can be characterized with relatively little effort. A totally unknown sequence may be a frustrating result: considerable effort will be needed to understand its function.

Sequence comparison is a nontrivial pursuit, and both statistical and biological considerations are involved. Statistically significant similarities (under some model and at some chosen level of significance) may be biologically meaningless. Sequence motifs that are statistically nonsignificant in similarity may encode the same function (this is likely to occur because the statistical model based on sequence alone is incomplete). In an area fraught with such difficulties, common sense and interpretation based on utility are paramount.

Sequence dissimilarity can range from identity, difference due to sequencing errors, difference due to population polymorphism (individual variants), and differences in multiple copies of a gene in a single individual (multigene families) to wide evolutionary divergence of genes in different organisms. Sequences that are similar due to common function may not share a common ancestral sequence in biological evolution. In general, ideas about the evolutionary relationships of sequences are not experimentally testable. Sequence homology (similarity due to descent from a common ancestor) is a hypothesis, not an observable fact, except in the case of microbial populations with high mutation rates and short

generation times, which may be studied experimentally through time.

## 2 ANALYSIS OF SINGLE SEQUENCES

### 2.1 DNA COMPOSITION, ISOCHORES, AND CODON USAGE

Nucleotides in DNA sequences may be counted as singlets, doublets, or triplets in either strand. Doublets or triplets may be counted as overlapping or nonoverlapping in two or three phases, respectively, on either strand. The genomes of various organisms vary considerably in their DNA composition. Warm-blooded vertebrates have a higher G+C content, which correlates with the higher thermal stability of GC over AT base pairs. Composition of regions within a genome can also vary considerably. Mammalian genomes contain relatively GC-rich and AT-rich regions, which are called isochores. Overlapping doublet frequencies are highly characteristic for an organism. CG dinucleotides are less common than expected in vertebrates and angiosperms, probably because spontaneous deamination of 5-methylcytosine to thymine prevents the repair of methylated CpG. In DNA coding for protein, one phase of nonoverlapping triplets will be the phase of translation and the triplets will be codons. In a gene, the possible codons for each amino acid are unevenly used, and the frequency table for the 64 triplets is called codon usage. Codon usage is different between different species and between highly and lowly expressed gene in the same species.

### 2.2 MAPPING DNA SEQUENCE FEATURES

Mapping the position of features on a DNA sequence is an important step in investigating its function. It is easy to map sites that can be precisely defined, such as stop codons or restriction enzyme recognition sites. Once DNA has been sequenced, the sizes of the fragments produced with any enzyme can be readily calculated. Features such as promoters, splice junctions, and ribosome binding sites are very difficult to predict because they are hard to specify. Mapping is most simply achieved by comparing the probe sequence with each position of the DNA sequence in turn and noting the hits. More sophisticated algorithms exist for rapid searching in large problems.

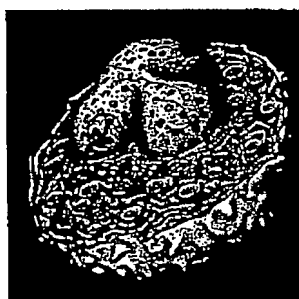
### 2.3 REPETITIVE SEQUENCES

Direct repeats and inverted repeats (sometimes called dyad symmetries) are common in DNA from many sources. Mammalian genomes contain families of long (LINE) and short (SINE) repeats. Repeats of *L1* (*Kpn* I) type are 5000 to 7000 bp long and are present in the genome in  $10^3$  to  $10^4$  copies. Repeats of *Alu* type are 350 bp long and occur in as many as  $9 \times 10^4$  copies. *Alu* repeats make human DNA hard to assemble from gel sequencing reads into the finished sequence. Inverted repeats occur in DNA coding for structural RNA, and these symmetry properties enable the RNA to fold into its secondary structure.

The dot plot is a diagram that reveals the presence of repeats and inverted repeats in sequences. It is also useful for comparing two different nucleic acid or protein sequences to detect regions of similarity. The dot plot is a rectangular array with rows labeled by one sequence and columns labeled by the other. A cell  $i, j$  can be used to represent the result of comparison of the  $j$ th residue of sequence A with the  $i$ th residue of sequence B. The simplest form of dot plot results from placing a diagonal mark in each cell where

# Molecular Cell Biology

SECOND EDITION



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ent amino acids in proteins. Thus a 100-unit protein has  $20^{100}$  (more than  $10^{130}$ ) possible structures. This enormous variability means that cells and organisms can differ greatly in structure and function even though they are constructed of the same types of biopolymers produced by similar chemical reactions.

Starch (a storage form of glucose in plant cells), cellulose (a constituent of plant cell walls), and glycogen (a storage form of glucose in liver and muscle cells) are examples of another important type of biopolymer: the polysaccharide, which is built of sugar monomers (Figure 2-1). At least 15 different monomeric sugars can be bonded in multiple ways to form various polysaccharides; thus many polysaccharides are nonlinear, branched molecules.

Monomers are not the only small molecules important to cell structure. The lipids, for example, form the basic structure of cell membranes. Lipids cohere noncovalently in very large sheetlike complexes; the membranes thus formed are as crucial to living systems as are the biopolymers.

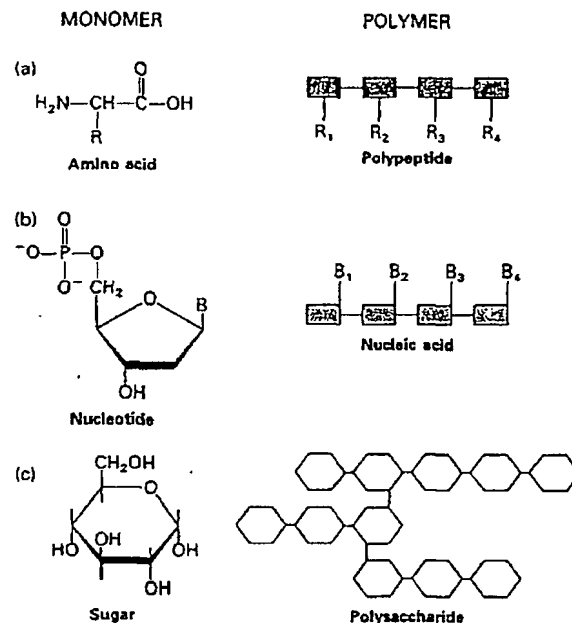
This chapter deals with the structures and some functions of biopolymers and small molecules; later chapters describe how the polymers are made and consider many of their other functions and interactions. ▲

## Proteins

Proteins are the working molecules of the cell. They catalyze an extraordinary range of chemical reactions, provide structural rigidity, control membrane permeability, regulate the concentrations of metabolites, recognize and noncovalently bind other biomolecules, cause motion, and control gene function. These incredibly diverse tasks are performed by molecules constructed from only 20 different amino acids.

### Amino Acids—the Building Blocks of Proteins—Differ Only in Their Side Chains

The monomers that make up proteins are called amino acids because, with one exception, each contains an amino group ( $-\text{NH}_2$ ) and an acidic carboxyl group ( $-\text{COOH}$ ). The exception, proline, has an imino group ( $-\text{NH}-$ ) instead of an amino group. At typical pH values in cells, the amino and carboxyl groups are ionized as  $-\text{NH}_3^+$  and  $-\text{COO}^-$ . All amino acids are constructed according to a basic design: a central carbon atom, called the  $\alpha$  carbon  $\text{C}_\alpha$  (because it is adjacent to the acidic carboxyl group), is bonded to an amino (or imino) group, to the carboxyl group, to a hydrogen atom, and to one variable group, called a side chain or R group (Figure 2-2). The side chains give the amino acids their individuality.



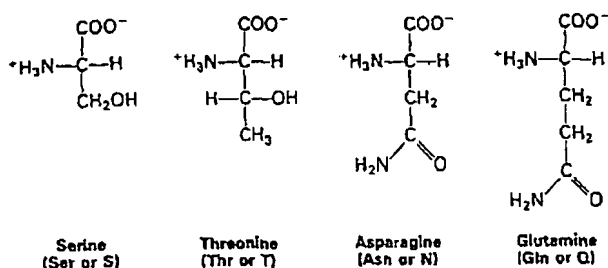
▲ **Figure 2-1** (a) Proteins, linear biopolymers called polypeptides, are formed from monomeric subunits termed amino acids. Each of the 20 different amino acids has a different R group, or side chain. Thus the polypeptide shown here, which is constructed of four amino acids, has  $20^4$ , or 160,000, possible structures. (b) Nucleic acids, also linear biopolymers, are formed from four monomers termed nucleotides, each of which has a different nitrogen-containing base structure (B). The nucleic acid shown here has  $4^4$ , or 256, possible structures. (c) Polysaccharides are built of monomeric saccharide (sugar) subunits. Because sugar residues can bind to one another at different positions, nonlinear branching polymers are often formed. The rings in (b) and (c) are depicted as Haworth projections (planar structures with a hint of perspective).

The amino acids represent the alphabet in which linear proteins are “written”; any student of biology must be familiar with the special properties of each letter of this alphabet. These letters can be classified into a few distinct categories.

The side chains of four of the amino acids are highly ionized and therefore charged at neutral pH. Arginine and lysine are positively charged; aspartic acid and glutamic acid are negatively charged and exist as aspartate and glutamate. The side chain of a fifth amino acid, histidine, is positively charged, but only weakly at neutral pH. In many cases, arginine may substitute for lysine, or aspartate for glutamate, with little effect on the structure or function of the protein.

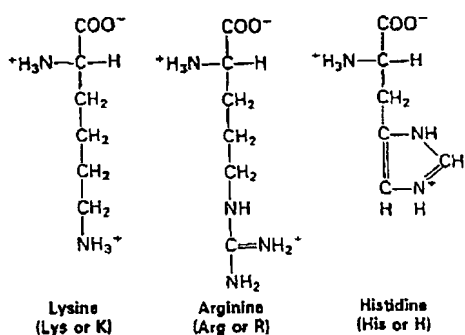
Serine and threonine, whose side chains have an  $-\text{OH}$  group, can interact strongly with water by forming hydrogen bonds. The side chains of asparagine and gluta-

## POLAR BUT UNCHARGED R GROUPS

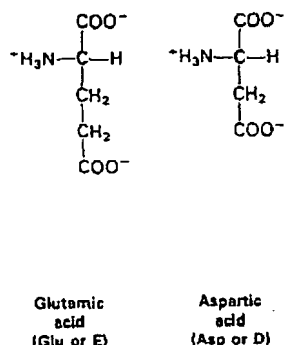


▼ Figure 2-2 The structures of the 20 common amino acids. In each structure, a central carbon atom (the  $\alpha$  carbon) is bonded to an amino group (or to an imino group in proline), a carboxyl group, a hydrogen atom, and an R group. The R groups are in red.

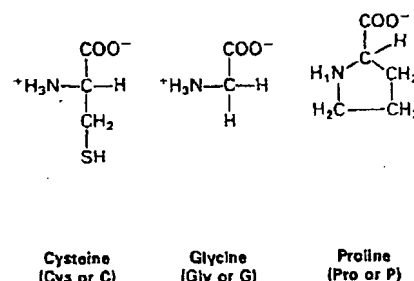
## POSITIVELY CHARGED R GROUPS



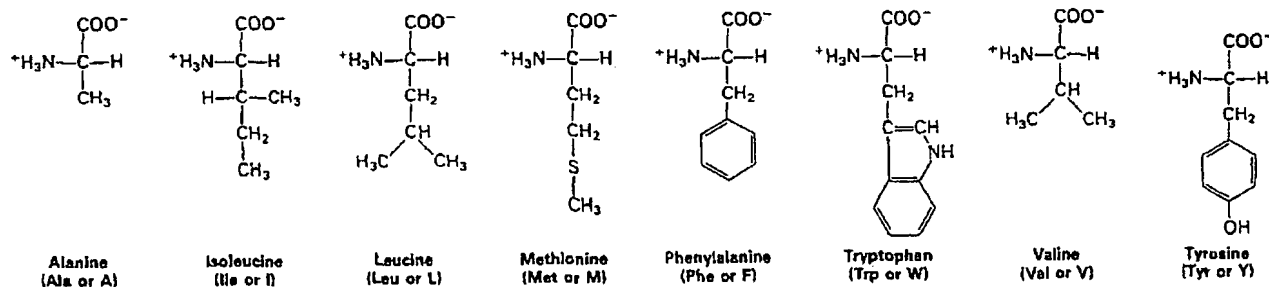
## NEGATIVELY CHARGED R GROUPS



## SPECIAL AMINO ACIDS



## HYDROPHOBIC R GROUPS



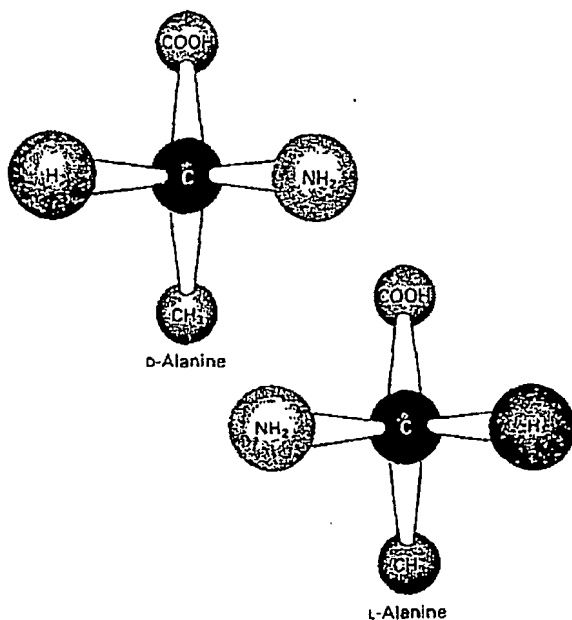
mine have polar amide groups with even more extensive hydrogen-bonding capacities. Together with the charged amino acids, these amino acids constitute the nine hydrophilic or polar amino acids.

The side chains of several other amino acids—alanine, isoleucine, leucine, methionine, phenylalanine, tryptophan, and valine—consist only of hydrocarbons, except for the sulfur atom in methionine and the nitrogen atom in tryptophan. These nonpolar amino acids are hydrophobic; their side chains are only slightly soluble in water. Tyrosine is also strongly hydrophobic because of its benzene ring, but its hydroxyl group allows it to interact with water, making its properties somewhat ambiguous.

Cysteine plays a special role in proteins because its —SH group allows it to dimerize through an —S—S— bond to a second cysteine, thus covalently linking regions of polypeptide to one another. When the —SH remains free, cysteine is quite hydrophobic.

Two other special amino acids are glycine and proline. Glycine has a hydrogen atom as its R group; thus it is the smallest amino acid and has no special hydrophobic or hydrophilic character. Proline, as an imino acid, is very rigid and creates a fixed kink in a polypeptide chain. It is quite hydrophobic.

The structure of all amino acids except glycine are asymmetrically arranged around the  $\alpha$  carbon, because it is bonded to four different atoms or groups of atoms

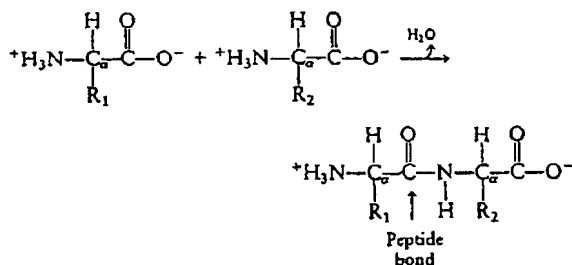


▲ Figure 2-3 Stereoisomers of the amino acid alanine. The  $\alpha$  carbon is black.

( $-\text{NH}_2$ ,  $-\text{COOH}$ ,  $-\text{H}$ , and  $-\text{R}$ ). Thus all amino acids except glycine can have one of two stereoisomeric forms. By convention, these mirror-image structures are called the D and the L forms of the amino acid (Figure 2-3). They cannot be interconverted without breaking a chemical bond. With rare exceptions, only the L forms of amino acids are found in proteins.

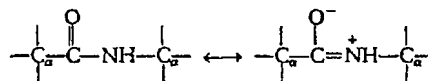
### Polypeptides Are Polymers Composed of Amino Acids Connected by Peptide Bonds

The *peptide bond*, the chemical bond that connects two amino acids in a polymer, is formed between the amino group of one amino acid and the carboxyl group of another. This reaction, called *condensation*, liberates a water molecule:



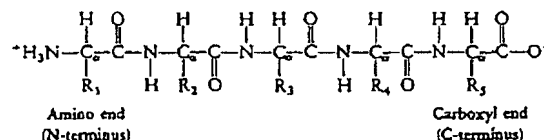
Because the carboxyl carbon and oxygen atoms are connected by a double bond, the peptide bond between car-

bon and nitrogen exhibits a partial double-bond character, as shown by the resonance structures



making it shorter than the typical C—N single bond. The six atoms of the peptide group (the two carbons of the adjacent amino acids and the carbon, oxygen, nitrogen, and hydrogen atoms of the bond) lie in the same plane (Figure 2-4a). However, adjacent peptide groups are not necessarily coplanar, due to rotation about the C—C $_\alpha$  and N—C $_\alpha$  bonds (Figure 2-4b).

A single linear array of amino acids connected by peptide bonds is called a *polypeptide*. If the polypeptide is short (fewer than 30 amino acids long), it may be called an *oligopeptide* or just a *peptide*. Polypeptides in living cells differ greatly in length; they generally contain between 40 and 1000 amino acids. Each polypeptide has a free amino group at one end (the N-terminus) and a free carboxyl group at the other (the C-terminus):

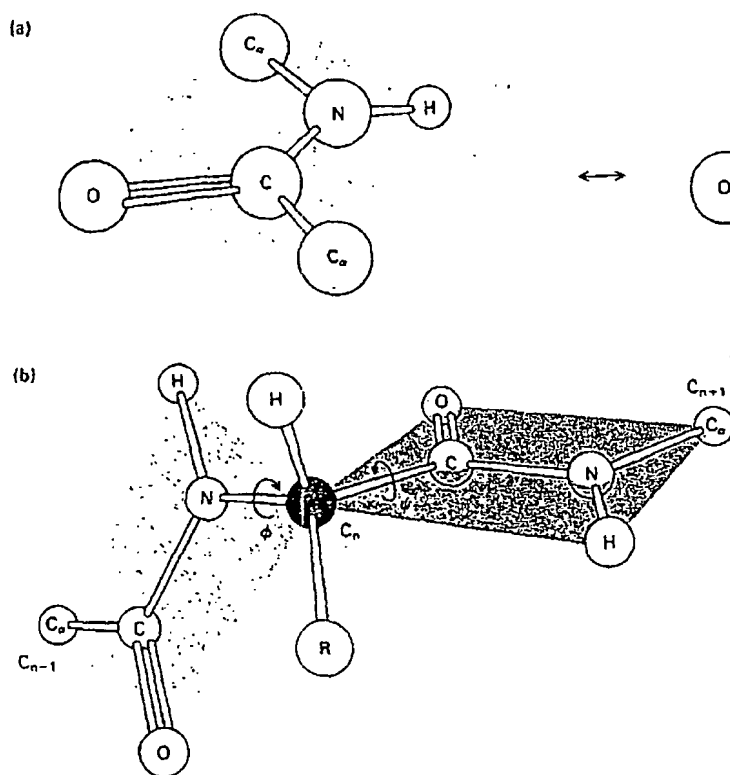


A protein is not merely a linear string of amino acids. The polypeptide folds up to form a specific three-dimensional structure that can be a long rod, as in the *fibrous proteins* that give tissues their rigidity, or a compact ball called a *globular protein*, as in many proteins that catalyze chemical reactions (enzymes), or a combination of balls and rods. The polypeptide can be modified further by the covalent or noncovalent attachment of additional small molecules.

A protein adopts a stable, folded conformation mainly through noncovalent (ionic, hydrogen, van der Waals, and hydrophobic) interactions. Its stability is also enhanced by the formation of covalent disulfide bonds between cysteines in different parts of the chain. Proteins may also consist of multiple polypeptide chains held together by noncovalent forces and, in some cases, by disulfide bonds. A well-characterized example is the hemoglobin molecule, which consists of four chains: two identical  $\alpha$  chains and two identical  $\beta$  chains (Figure 2-5).

### Three-dimensional Protein Structure Is Determined through X-ray Crystallography

The detailed three-dimensional structures of numerous proteins have been established by the painstaking efforts of many workers—notably, Max Perutz and John Kendrew, who perfected the x-ray crystallography of



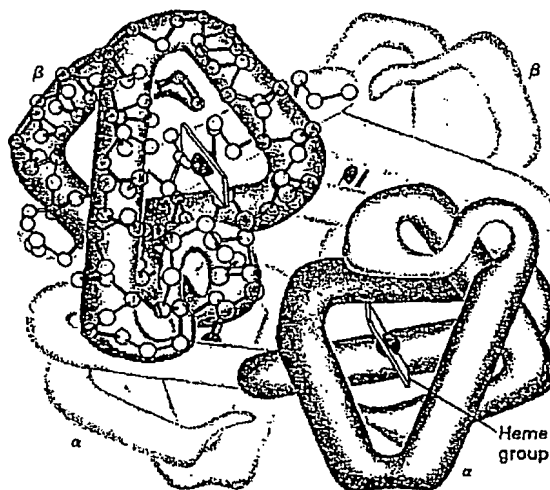
◀ **Figure 2-4** (a) Because the carbon-nitrogen peptide bond has a partial double-bond character, the peptide group is planar. (b) However, there is considerable flexibility in the geometry of polypeptides: rotation is possible about the two covalent single bonds that connect each  $\alpha$  carbon to the two adjacent planar peptide units. But some restrictions do apply to the values of  $\psi$  and  $\phi$ . For example, if the pictured adjacent peptide groups were coplanar, then certain oxygen and hydrogen atoms would be separated by less than their van der Waals radii and would repel one another.

proteins, in which beams of x-rays are passed through a crystal of protein. The wavelengths of x-rays are about 0.1–0.2 nanometers (nm)—short enough to resolve the atoms in the protein crystal. The three-dimensional structure of the protein can be deduced from the *diffraction pattern* of discrete spots that is produced when the scattered radiation is intercepted by photographic film. Such patterns are extremely complex; as many as 25,000 diffraction spots can be obtained from a small protein. Elaborate calculations and modifications of the protein (such as binding of heavy metal) must be made to interpret the diffraction pattern and to solve the structure of the protein.

Recently, three-dimensional structures of some small proteins have been determined by nuclear magnetic resonance (nmr) methods. An advantage of this approach is that it avoids the need to crystallize the protein. A disadvantage is that it is limited to relatively small proteins (up to about 20,000 molecular weight).

### The Structure of a Polypeptide Can Be Described at Four Levels

The structures adopted by polypeptides can be divided into four levels of organization. *Primary structure* refers to the linear arrangement of amino acid residues along a



▲ **Figure 2-5** The conformations assumed by the two  $\alpha$  and two  $\beta$  chains in a molecule of hemoglobin. Each chain forms several  $\alpha$  helices (see Figure 2-6). Only the backbones formed by the carbon and nitrogen atoms of the chains are shown here. A multitude of noncovalent interactions stabilize the conformations of the individual chains and the contacts between them. A heme group is bound to each chain. After R. E. Dickerson and I. Geis, 1969, *The Structure and Action of Proteins*, Benjamin-Cummings, p. 56. Copyright 1969 by Irving Geis.

polypeptide chain and to the locations of covalent bonds (mainly —S—S— bonds) between chains. *Secondary structure* pertains to the folding of parts of these chains into regular structures, such as  $\alpha$  helices and  $\beta$  pleated sheets. *Tertiary structure* includes the folding of regions between  $\alpha$  helices and  $\beta$  pleated sheets, as well as the combination of these secondary features into compact shapes (domains). *Quaternary structure* refers to the organization of several polypeptide chains into a single protein molecule, such as in hemoglobin.

### Two Regular Secondary Structures Are Particularly Important

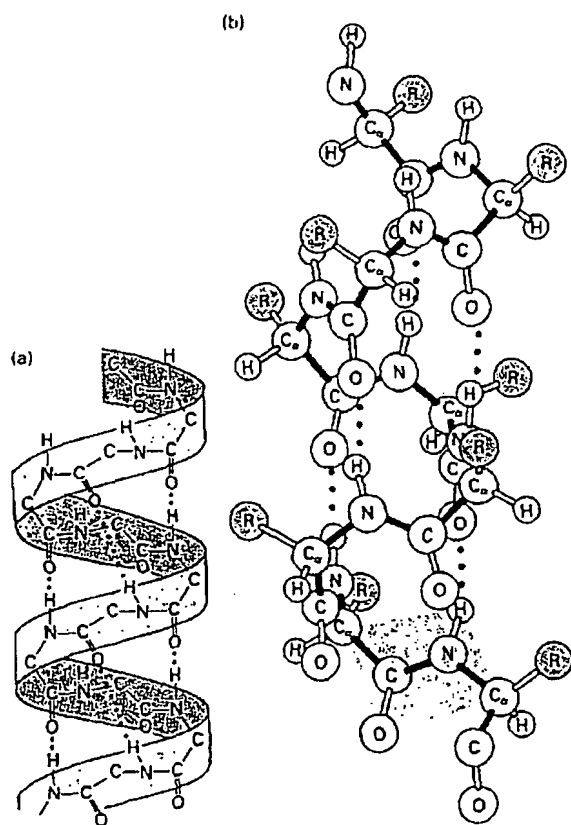
**The  $\alpha$  Helix** Although some regions of proteins are held in unique and irregular conformations, much protein structure involves repeated use of a limited number of regular configurations. One common structure, the  $\alpha$  helix, was first described by Linus Pauling and Robert B. Corey in 1951. Through careful model building, these scientists came to realize that polypeptide seg-

ments composed of certain amino acids tend to arrange themselves in regular helical conformations. In an  $\alpha$  helix, the carboxyl oxygen of each peptide bond is hydrogen-bonded to the hydrogen on the amino group of the fourth amino acid away (Figure 2-6), so that the helix has 3.6 amino acids per turn. Each amino acid residue represents an advance of about 1.5 Å along the axis of the helix. Every C=O and N—H group in the peptide bonds participates in a hydrogen bond, and the rigid planarity of the peptide bonds contributes to the rigid shape of the helix. In this inflexible, stable arrangement of amino acids, the side chains are positioned along the outside of a cylinder. The hydrogen-bonding potential of the peptide bonds is entirely satisfied internally, so that the polar or nonpolar quality of the cylindrical surface is determined entirely by the side chains. At least some of the amino acids in most proteins are organized into  $\alpha$  helices.

Certain amino acid sequences adopt the  $\alpha$ -helical conformation more readily than others. What determines this propensity is complicated, but some simple factors are evident. For instance, proline is rarely found in  $\alpha$ -helical regions because it cannot use its peptide nitrogen to make a hydrogen bond. Glycine also is an infrequent participant. Another inhibiting factor can be the tendency of multiple charged residues to repel each other.

The  $\alpha$  helix is a rodlike element of protein structure that serves many functions. A globular protein can be made up of short  $\alpha$ -helical rods connected by bends that allow the rods to interact with each other; hemoglobin, for instance, is 70 percent  $\alpha$  helical (see Figure 2-5). Alternatively, a single rod can span a long distance, as in the protein on the surface of the influenza virus (Figure 2-7a). Even in extended molecules, a, b, c the  $\alpha$  helix is usually found packed against other elements of protein, not as an isolated structure. Long fibers, such as the skin protein keratin or the muscle protein myosin (Figure 2-7b), can be formed by two or three  $\alpha$  helices that wrap gently around each other to form *coiled coils*. Small rods of  $\alpha$  helix interact with DNA in some DNA-binding proteins (Figure 2-7c). A helical rod bearing only hydrophobic side chains can span lipid membranes well because the hydrophilic peptide bonds are buried inside the helix.

Many  $\alpha$  helices are *amphipathic*: they expose hydrophilic side chains on one face and hydrophobic side chains on another face. Looking down the central axis of an  $\alpha$  helix (Figure 2-8a), the amino acid residues are arranged in a wheel; if the helix is amphipathic, most or all



◀ **Figure 2-6** Models of the  $\alpha$  helix. (a) This ribbonlike representation without R groups emphasizes the helical form. (b) This ball-and-stick representation emphasizes the role of the individual atoms and shows the R groups (green) that protrude from the helix body at regular intervals. Some of the planes of the  $C_\alpha$ -CO-NH groups are shaded orange. Part (b) after L. Stryer, 1988, *Biochemistry*, 3d ed., W. H. Freeman and Company, p. 26.

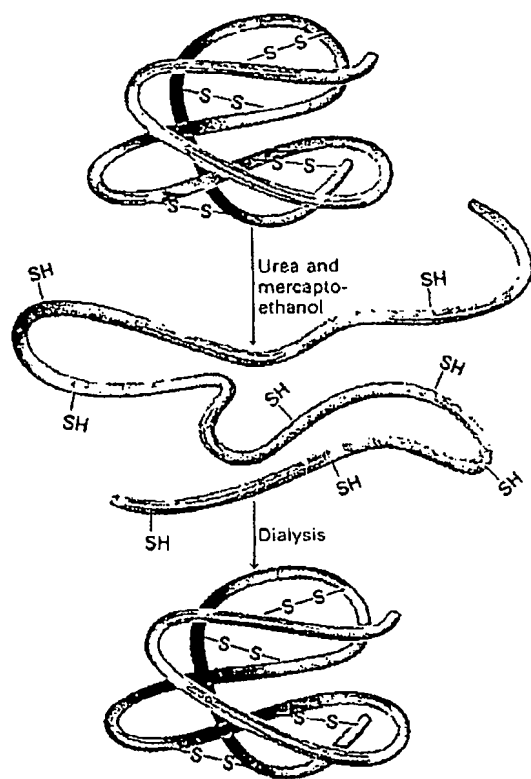


valently bound prosthetic group. For example, staphylococcal nuclease—a bacterial enzyme of 149 residues that degrades DNA and RNA—is totally denatured in acid but renatures to its native conformation within 0.1 s after the solution is neutralized. The three-dimensional architecture of this protein is solely a consequence of interactions among its amino acids and with its aqueous environment. In such cases, the genetic program of the cell must only define the primary structure of the protein—the amino acid sequence—and the tertiary structure is assured. With care, most proteins can be carried through a denaturation-renaturation cycle. Thus it is generally true that linear structure determines three-dimensional architecture.

The native form of some proteins is not the conformation with the lowest free energy and consequently cannot be completely restored on renaturation. This is particu-

larly true of multichain proteins. The two chains of insulin, for example, can be separated by a combination of reducing agents (to break the disulfide bridges) and concentrated solutions of such chemicals as urea (to disrupt hydrogen and hydrophobic bonds). When the insulin renatures in the presence of oxidizing agents that promote the formation of disulfide bridges, a number of stable multichain aggregates do form, but *native* insulin molecules make up only a minor proportion of them. In the others, the re-formed disulfide bridges connect inappropriate parts of the chain.

Insulin is formed by the partial proteolysis (breaking down) of proinsulin, its larger precursor (see Figure 2-13). Denatured proinsulin, as opposed to the denatured two-chain form of insulin, can renature to form the native structure of proinsulin with a high efficiency. Presumably, within the cell, either proinsulin or preproinsulin folds in such a way that the correct disulfide bridges form at the lowest free energy. The cell utilizes these intermediate stages to form insulin, whose stable conformation is not the one of lowest free energy.



▲ **Figure 2-15** Denaturation and renaturation of a protein. Most polypeptides can be completely unfolded by treatment with an 8 M urea solution containing mercaptoethanol ( $\text{HSCH}_2\text{CH}_2\text{OH}$ ). The urea breaks intramolecular hydrogen and hydrophobic bonds, and the mercaptoethanol reduces each disulfide bridge to two  $\text{-SH}$  groups. When these chemicals are removed by dialysis, the  $\text{-SH}$  groups on the unfolded chain oxidize spontaneously to re-form disulfide bridges, and the polypeptide chain simultaneously refolds into its native configuration.

## Enzymes

Protein catalysts called *enzymes* are mediators of the dynamic events of life; almost every chemical reaction in a cell is catalyzed by an enzyme. Like other catalysts, enzymes increase the rates of reactions that are already energetically favorable; more precisely, enzymes increase the rates of forward and reverse reactions by the same factor. The name of an enzyme usually indicates its function: the suffix *-ase* is commonly appended to the name of the type of molecule on which the enzyme acts. Thus proteases degrade proteins, phosphatases remove phosphate residues, and ribonuclease cleaves RNA molecules.

The chemicals that undergo a change in a reaction catalyzed by an enzyme are the *substrates* of that enzyme. Because little free energy may be liberated in either direction in reversible reactions, the distinction between chemicals that are substrates and those that are products is often arbitrary.

Most enzymes are found inside cells, but a number are secreted by cells and function in the blood, the digestive tract, or other extracellular spaces. In microbial species, some enzymes function outside the organism. The number of different types of chemical reactions in any one cell is very large: an animal cell, for example, normally contains 1000–4000 different types of enzymes, each of which catalyzes a single chemical reaction or set of closely related reactions. Certain enzymes are found in the majority of cells because they catalyze common cellular reactions—the synthesis of proteins, nucleic acids, and phospholipids and the conversion of glucose and oxygen into carbon dioxide and water, which produces most of the chemical energy used in animal cells. Other enzymes are

found only in a particular type of cell within an organism, such as a liver cell or a nerve cell, because they carry out some chemical reaction unique to that cell. Also, many mature cells, including erythrocytes (red blood cells) and epidermal (skin) cells, may no longer be capable of making proteins or nucleic acids yet these cells still contain specific sets of enzymes that they synthesized at an earlier stage of differentiation.

### Certain Amino Acids in Enzymes Bind Substrates: Others Catalyze Reactions on the Bound Substrates

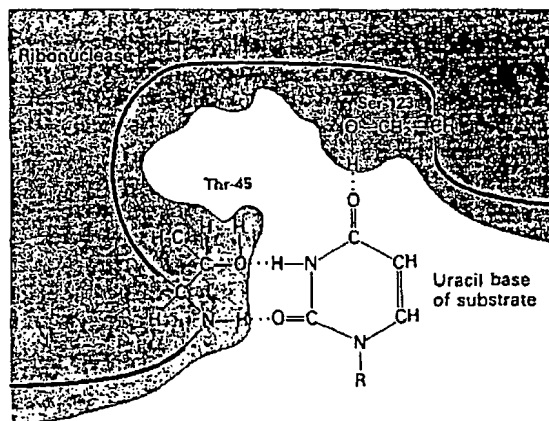
Two striking properties characterize all enzymes: their enormous *catalytic power* and their *specificity*. Quite often, the rate of an enzymatically catalyzed reaction is  $10^6$ – $10^{12}$  times that of an uncatalyzed reaction under otherwise similar conditions. The specificity of an enzyme is determined by the different rates at which it catalyzes closely similar chemical reactions or by its ability to distinguish between closely similar substrates.

Certain amino acid side chains of an enzyme are important in determining its specificity and its ability to accelerate the reaction rate. The properties of an enzyme are thus functions of its linear arrangement of amino acids and of the appropriate foldings of the peptide chain. Enzyme molecules have two important regions, or sites: one that recognizes and binds the substrate(s), and one that catalyzes the reaction once the substrate(s) have been bound. The amino acids in each of these key regions do not need to be adjacent in the linear polypeptide; they are brought into proximity in the folded molecule. In some enzymes, the catalytic site is part of the substrate-binding site. These two regions are called, collectively, the *active site*.

The binding of a substrate to an enzyme usually involves the formation of multiple noncovalent ionic, hydrogen, and hydrophobic bonds and van der Waals interactions (Figure 2-16). The array of chemical groups in the active site of the enzyme is precisely arranged so that the specific substrate can be more tightly bound than any other molecule (with the exception of some enzyme inhibitors) and the reaction can occur readily. In catalysis, covalent bonds between the enzyme and the substrate may be formed (and then broken) to reduce the activation energy for the reaction.

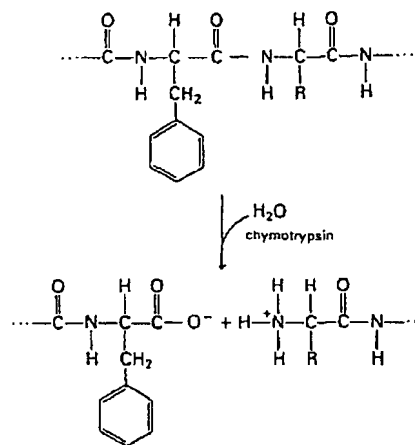
### Trypsin and Chymotrypsin Are Well-characterized Proteolytic Enzymes

The proteolytic (protein-digesting) enzymes trypsin and chymotrypsin are synthesized in the pancreas and secreted into the small intestine as inactive precursors, or *zymogens*, called trypsinogen and chymotrypsinogen, respectively. These zymogens are not activated until they reach the small intestine where they hydrolyze peptide

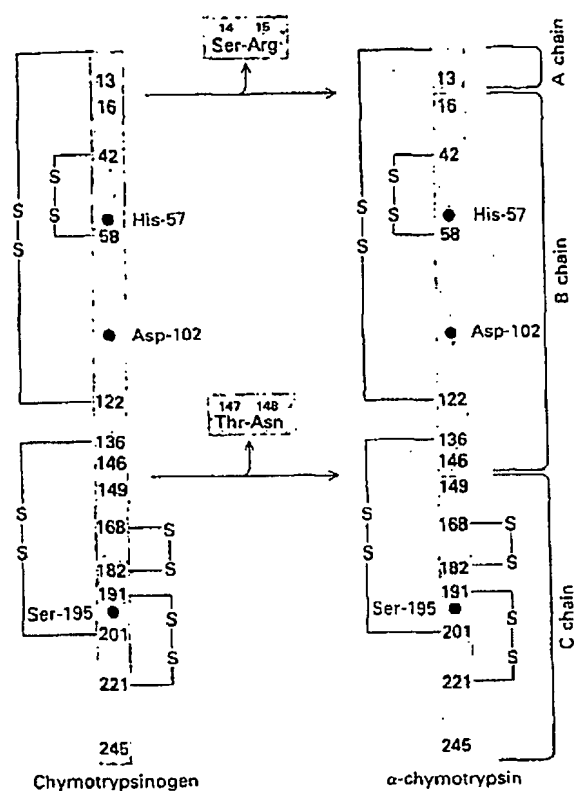


▲ **Figure 2-16** The specific binding of a substrate to an enzyme involves the formation of multiple noncovalent bonds. Here, two amino acid residues of the enzyme ribonuclease bind uracil, part of its substrate, by three hydrogen bonds. Substrates without the two C=O groups and one N—H group in the appropriate positions would be unable to bind or would bind less tightly. Other regions of the enzyme, not depicted here, bind other parts of the RNA substrate by hydrogen bonds and van der Waals interactions.

bonds of ingested proteins—a step in their digestion to single amino acids (Figure 2-17). The delay in activation serves an important regulatory purpose: it prevents the enzyme from digesting the pancreatic tissue in which it was made. Two irreversible proteolytic cleavages activate chymotrypsin. One cleavage removes serine 14 (the serine at position 14) and arginine 15 from chymotrypsinogen; the other removes threonine 147 and asparagine 148



▲ **Figure 2-17** The hydrolysis of a peptide bond by chymotrypsin.



▲ **Figure 2-18** A linear representation of the conversion of chymotrypsinogen into chymotrypsin by the excision of two dipeptides. The positions of the disulfide bridges are indicated. In the folded molecule, histidine 57, aspartate 102, and serine 195 are located in the active site.

(Figure 2-18). Removal of these two dipeptides activates the protease function of the enzyme.

The hydrolysis of peptide bonds is energetically favorable ( $\Delta G^\circ = -2$  kcal/mol). Nonetheless, the activation energy for an *uncatalyzed* peptide-bond hydrolysis—say, in a neutral aqueous solution of a protein at room temperature—is so high that there is little or no hydrolysis even after several months. Biochemists can chemically hydrolyze proteins into their constituent amino acids by treating them with a 6 M solution of hydrochloric acid in an evacuated tube at 100°C for 24 h. Yet at 37°C and neutral pH, a molecule of trypsin or chymotrypsin can catalyze the hydrolysis of up to 100 peptide bonds per second. The power of enzymatically mediated catalysis is well-illustrated here: the addition of sufficient enzyme can do in seconds what otherwise would require harsh conditions and long times.

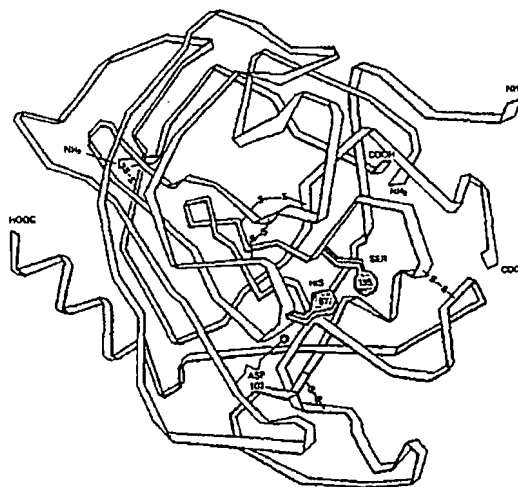
Chymotrypsin does not hydrolyze all peptide bonds; rather, it is selective for the peptide bond at the carboxyl ends of amino acids such as phenylalanine, tyrosine, and

tryptophan, which have large hydrophobic side chains. Trypsin, by contrast, is specific for the peptide bond on the C-terminal side of lysine and arginine residues.

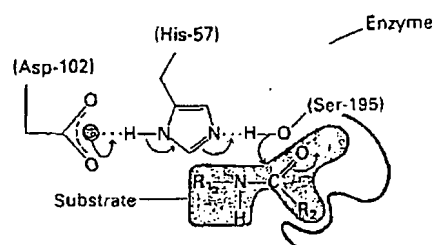
#### *Specific Amino Acid Side Chains of Chymotrypsin Aid in Substrate Binding*

The reaction mechanism of chymotrypsin was deduced, in part, from the three-dimensional structure obtained by x-ray crystallography (Figure 2-19). The enzyme contains three polypeptides—the A, B, and C chains, which have 13, 131, and 97 amino acids, respectively. These chains are interconnected by disulfide bridges (see Figures 2-18 and 2-19). The molecule has two key structural features: the active site and the *hydrophobic cleft* (a crevice bordered by the side chains of several hydrophobic amino acid residues), which serves as the binding site for specific amino acid residues on the substrate. The conformation of this pocket allows the residues lining it to participate in hydrophobic interactions with the large hydrophobic side chains of phenylalanine, tyrosine, or tryptophan. Neither charged side chains nor small hydrophobic residues on the substrate can make the noncovalent bonds necessary to fit into the cleft.

The hydrophobic residues of most globular proteins are buried in the interior; when such proteins are in their native states, the peptide bonds linking the hydrophobic residues are not accessible to hydrolysis by chymotrypsin. Normally, stomach acids (pH 1) denature ingested proteins so that proteases in that organ can partly degrade them before their exposure to further digestion by chymotrypsin at neutral pH in the intestine.

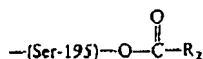


▲ **Figure 2-19** A three-dimensional model of  $\alpha$ -chymotrypsin determined from x-ray analysis. The N- and C-termini of the A, B, and C chains are indicated, as are the —S—S— bridges and the three amino acid residues of the active site (red). After B. W. Matthews et al., 1967, *Nature* 214:652.



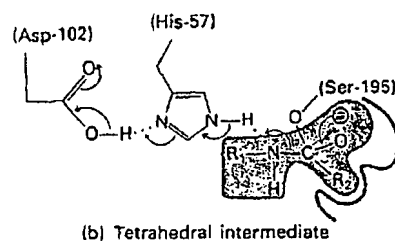
(a) Enzyme-substrate complex

**▲ Figure 2-20** The mechanism of hydrolysis of a peptide bond by  $\alpha$ -chymotrypsin. Red curved arrows represent the movement of electrons. (a) The substrate is bound to the enzyme so that the bond to be hydrolyzed is positioned near serine 195. The negative charge (blue) surrounding the oxygens in aspartate 102 induces a charge relay system, which is initiated when the oxygen atoms on Asp-102 attract a proton from the nitrogen atom on His-57. When the negative charge reaches the second nitrogen in His-57, the nitrogen removes the proton from the hydroxyl group on Ser-195. The resulting  $\text{O}^-$  attacks the carbon of the bound substrate to form (b) a tetrahedral intermediate, so called because the carbon atom of interest temporarily has four single bonds. The hydrogen bound to the second nitrogen in His-57 is then added to the nitrogen of the substrate. As a result, the C-N bond of the substrate breaks, leaving (c)  $\text{R}_1\text{NH}_2$  and the acylenzyme intermediate

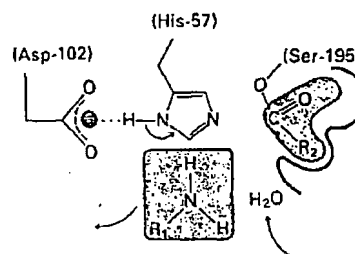


The  $\text{R}_1\text{NH}_2$  is discharged from the enzyme and replaced by water. In the resulting structure (d), a similar charge relay system is induced, and His-57 removes a proton from the hydrogen-bonded  $\text{H}_2\text{O}$ . The  $\text{OH}^-$  thus generated attacks the carbonyl carbon of the acylenzyme to form (e) another tetrahedral intermediate. The bond between the tetrahedral carbon and the oxygen of Ser-195 is hydrolyzed to yield (f)  $\text{R}_2\text{COO}^-$  bound noncovalently to the free enzyme, from which it is released. After R. M. Stroud, et al., 1975, in *Proteases and Biological Control*, E. Reich et al., eds. Cold Spring Harbor Laboratory, p. 25.

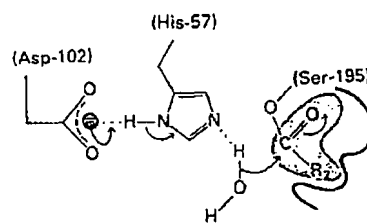
**Other Amino Acid Side Chains of Chymotrypsin Have Roles in Catalyzing the Hydrolysis of the Bound Substrate** The catalytic activity of chymotrypsin depends on three amino acid residues: histidine 57, aspartate 102, and serine 195. These amino acids are distant from one another in the primary structure of the protein (see Figure 2-18), but the chains are folded in such a way in the active enzyme molecule that the three side chains are close together, in the correct position for catalyzing the hydrolysis of a peptide bond in a protein bound to the enzyme (see Figure 2-19). When chymotrypsinogen is proteolytically activated, the polypeptide conformation is altered to bring these three residues into correct alignment.



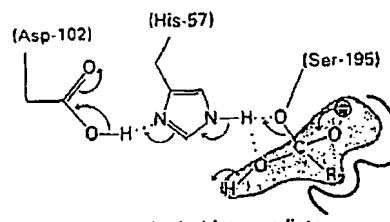
(b) Tetrahedral intermediate



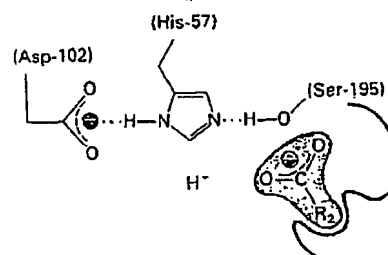
(c) Acylenzyme



(d) Acylenzyme

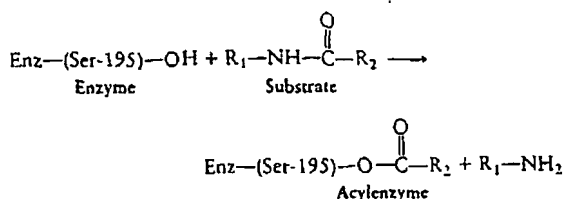


(e) Tetrahedral intermediate

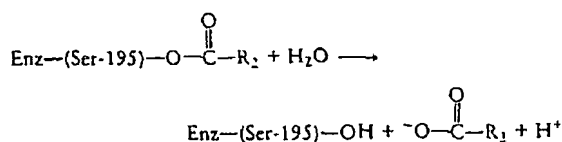


(f) Enzyme-product complex

The hydrolysis reaction proceeds in two main steps. First, the peptide bond is broken and the carboxyl group is transferred to the hydroxyl residue of serine 195:



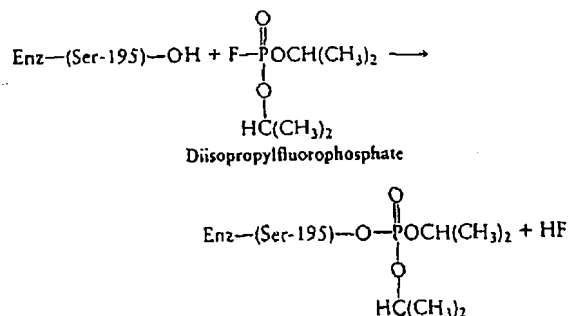
Second, this *acylenzyme* intermediate is hydrolyzed:



Note that the second step restores the enzyme to its original state.

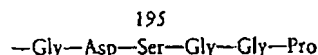
Aspartate 102 and histidine 57 facilitate the acylation reaction by removing the proton from serine 195 and adding it to the nitrogen of the departing amino group (Figure 2-20). In a similar manner, aspartate 102 and histidine 57 facilitate the hydrolysis of the acylenzyme. These enzymatically catalyzed steps—transfer of a proton from the enzyme to the substrate, formation of a covalent acylserine intermediate, and hydrolysis of the acylenzyme—all drastically reduce the overall activation energy of the proteolysis reaction.

The hydroxyl group on serine 195 is unusually reactive. The concept of an “active” serine residue at the active site predated the determination of the crystal structure of chymotrypsin. It was already known, for example, that the compound diisopropylfluorophosphate is a potent inhibitor of chymotrypsin; it reacts only with the hydroxyl on serine 195 to form a stable covalent compound that irreversibly inactivates the enzyme:



**Trypsin and Chymotrypsin Have Different Substrate-binding Sites** A comparison of trypsin and chymotrypsin will emphasize the nature of the specificity of enzymatically catalyzed reactions. About 40 percent of the amino acids in these two molecules are the same; in

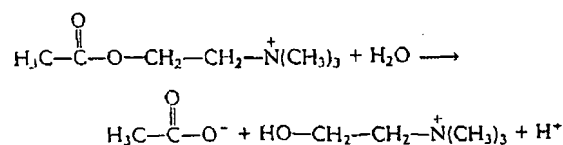
particular, the amino acid sequences in the vicinity of the key serine residue are identical:



The three-dimensional structures and catalytic mechanisms of these two enzymes are also quite similar, indicating that they evolved from a common polypeptide. The major difference between trypsin and chymotrypsin is found in the side chains of the amino acids that line the substrate-binding site. The negatively charged amino acids in this area of the trypsin molecule facilitate the binding of only positively charged (lysine or arginine) residues, instead of hydrophobic ones.

### Other Hydrolytic Enzymes Contain Active Serine

Other, mostly unrelated, hydrolytic enzymes also contain an active serine residue that is essential for catalysis. For example, acetylcholinesterase catalyzes the hydrolysis of the neurotransmitter acetylcholine to acetate and choline:



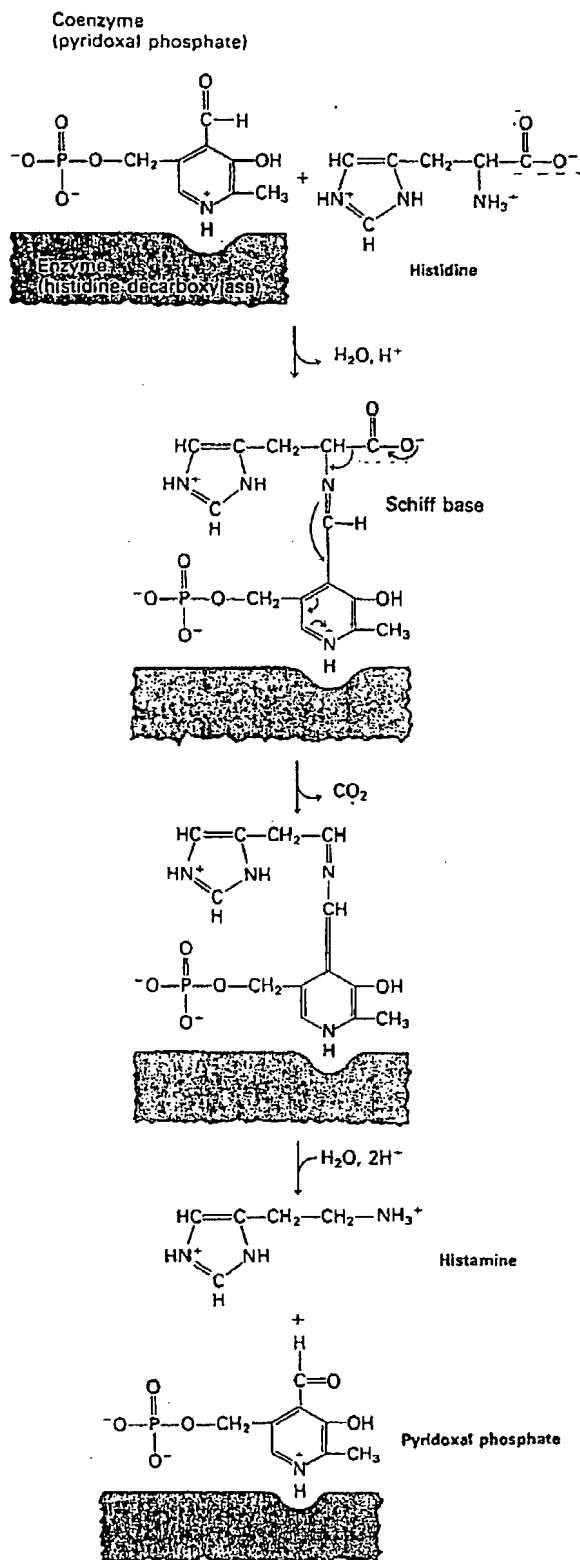
Diisopropylfluorophosphate is a potent, irreversible inhibitor of acetylcholinesterase as well as of chymotrypsin. The compound is lethal to animals because it blocks nerve transmission by causing a buildup of the transmitter substance. (The action of this transmitter is discussed in Chapter 20.)

### Coenzymes Are Essential for Certain Enzymatically Catalyzed Reactions

Many enzymes contain a *coenzyme*—a tightly bound small molecule or prosthetic group essential to enzymatic activity. Vitamins required in trace amounts in the diet are often converted to coenzymes. Coenzyme A, for instance, is derived from the vitamin pantothenic acid; the coenzyme pyridoxal phosphate is derived from vitamin B<sub>6</sub>. To cite just one example of how coenzymes function, we consider pyridoxal phosphate. The aldehyde group



can form a covalent complex called a *Schiff base* with an  $-\text{NH}_2$  group of an amino acid, which facilitates or lowers the activation energy for the breaking of bonds to the carbon of the amino acid. Figure 2-21 shows how pyridoxal phosphate catalyzes the decarboxylation of histidine to form histamine—a potent dilator of small blood vessels. Histamine is released by certain cells in the course of allergic hypersensitivity.



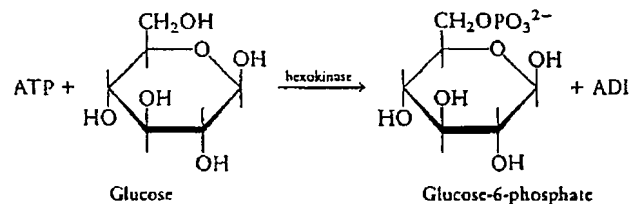
◀ **Figure 2-21** Pyridoxal phosphate, a coenzyme, participates in many reactions involving amino acids. When it is bound to histidine decarboxylase, as in this example, it forms a Schiff base with the  $\alpha$  amino group of histidine. The positive charge on the nitrogen of pyridoxal phosphate then attracts the electrons from the carboxylate group of the histidine, via a charge relay system. This weakens the bond between the  $\alpha$  carbon of the histidine and the carboxylate group, causing the release of  $\text{CO}_2$ . Finally, histamine, the reaction product, is hydrolyzed from the pyridoxal complex.

### Substrate Binding May Induce a Conformational Change in the Enzyme

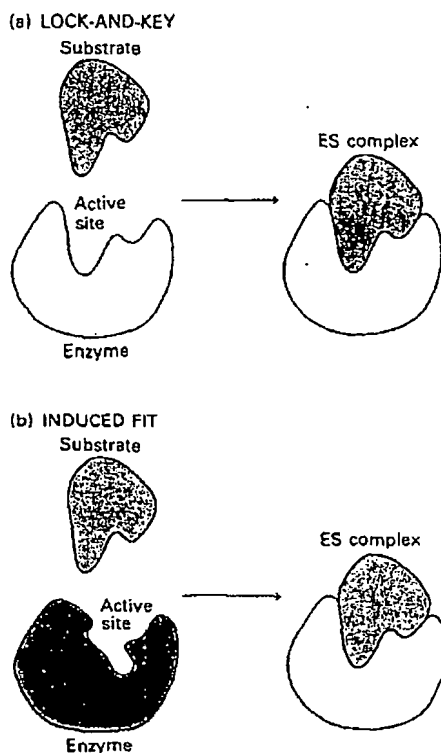
When a substrate binds to an enzyme, molecules of complementary charge or shape, or both, may simply fit together into a complex stabilized by a variety of noncovalent bonds. Such an interaction resembles the fitting of a key into a lock and is said to occur by a *lock-and-key* mechanism (Figure 2-22a).

In some enzymes, the binding of the substrate induces a conformational change in the enzyme that causes the catalytic residues to become positioned correctly. Molecules that attach to the substrate-binding site, or *recognition site*, of the enzyme but that do not induce a conformational change are not substrates of that enzyme. Thus an enzyme differentiates between a substrate and a nonsubstrate in two ways: Does the potential substrate bind to the enzyme? If so, does it induce the correct conformational change? When both criteria are met, the enzyme-substrate complex is said to demonstrate *induced fit* (Figure 2-22b).

An important example of induced fit is provided by the enzyme hexokinase, which catalyzes the transfer of a phosphate residue from ATP to a specific carbon atom of glucose:



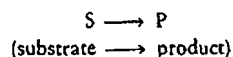
This is the first step in the degradation of glucose by cells. X-ray crystallography has shown that hexokinase consists of two domains. The binding of glucose induces a major conformational change that brings these domains closer together and creates a functional catalytic site (Figure 2-23). Only glucose and closely related molecules can induce this conformational change, ensuring that the enzyme is used to phosphorylate only the correct substrates. Molecules such as glycerol, ribose, and even water may bind to the enzyme at the recognition site but cannot induce the requisite conformational change, so they are not substrates for the enzyme.



▲ **Figure 2-22** Two mechanisms for the interaction of an enzyme and a substrate. (a) In the lock-and-key mechanism, the substrate fits directly into the binding site of the enzyme. (b) If binding occurs by induced fit, the substrate induces a conformational change in the enzyme that appropriately positions the substrate for catalysis.

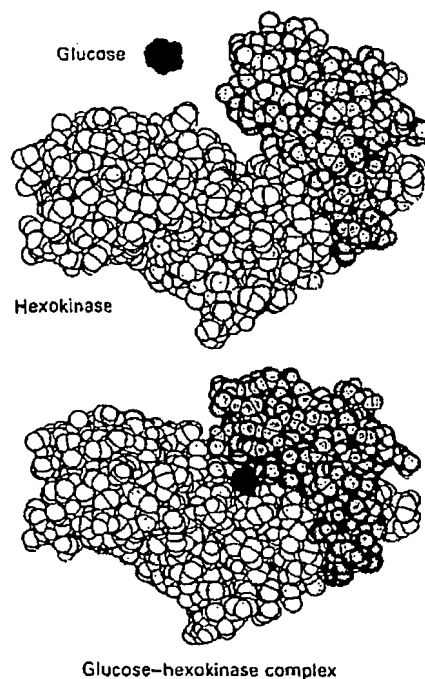
### The Catalytic Activity of an Enzyme Can Be Characterized by a Few Numbers

Enzymatic specificity is usually quantified by discrimination ratios: a good substrate may be cleaved 10,000 times as fast as a poor substrate. The catalytic power of an enzyme on a given substrate involves two numbers:  $K_m$ , which measures the affinity of the enzyme for its substrate, and  $V_{max}$ , which measures the maximal velocity of enzymatic catalysis. Equations for  $K_m$  and  $V_{max}$  are most easily derived by considering the simple reaction



in which the rate of product formation depends on  $[S]$ , the concentration of the substrate, and on  $[E]$ , the concentration of the catalytic enzyme. For an enzyme with a single catalytic site, Figure 2-24(a) shows how  $d[P]/dt$ , the rate of product production, depends on  $[S]$  when  $[E]$  is kept constant.

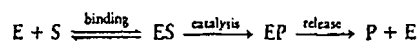
At low concentrations of  $S$ , the reaction rate is propor-



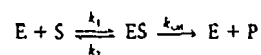
▲ **Figure 2-23** The conformation of hexokinase changes markedly when it binds the substrate glucose: the two domains of the enzyme come closer together to surround the substrate. Molecules such as the five-carbon sugar ribose can also bind to hexokinase by forming specific hydrogen bonds with groups in the substrate-binding pocket of the enzyme, but only glucose can form all of the bonds that cause the enzyme to change its conformation. Courtesy of Dr. Thomas A. Steitz.

tional to  $[S]$ ; as  $[S]$  is increased the rate does not increase indefinitely in proportion to  $[S]$  but eventually reaches  $V_{max}$ , at which it becomes independent of  $[S]$ .  $V_{max}$  is proportional to  $[E]$  and to a catalytic constant  $k_{cat}$  that is an intrinsic property of the individual enzyme; halving  $[E]$  reduces the rate at all values of  $[S]$  by one-half.

When interpreting curves such as those in Figure 2-24, bear in mind that all enzymatically catalyzed reactions include at least three steps: (1) the binding of the substrate ( $S$ ) to the enzyme ( $E$ ) to form an enzyme-substrate complex ( $ES$ ); (2) the conversion of  $ES$  to the enzyme-product complex ( $EP$ ); and (3) the release of the product ( $P$ ) from  $EP$ , to yield free  $P$ :



In the simplest case, the release of  $P$  is so rapid that we can write



The reaction rate  $d[P]/dt$  is proportional to the concentration of ES and to the catalytic constant  $k_{cat}$  for the given enzyme:

$$\frac{d[P]}{dt} = k_{cat} [ES] \quad (1)$$

To calculate [ES], we assume the reaction is in a steady state, so that  $k_1 [E] [S]$ , the formation rate of [ES], is equal to the rate of its consumption, either by dissociation of uncatalyzed substrate at a rate of  $k_2 [ES]$  or by catalysis at a rate of  $k_{cat} [ES]$ :

$$k_1 [E] [S] = (k_2 + k_{cat}) [ES] \quad (2)$$

If

$$[E]_{tot} = [E] + [ES] \quad (3)$$

(where  $[E]_{tot}$  is the sum of the free and the complexed enzyme, or the total amount of enzyme), then we can combine equations (2) and (3) to obtain

$$\begin{aligned} [E]_{tot} &= [E] + [ES] = \frac{(k_2 + k_{cat})}{k_1 [S]} [ES] + [ES] \\ &= [ES] \left[ 1 + \left( \frac{k_2 + k_{cat}}{k_1} \right) \left( \frac{1}{[S]} \right) \right] \end{aligned}$$

If we define  $K_m$ , called the *Michaelis constant*, as

$$\frac{k_2 + k_{cat}}{k_1} \quad (4)$$

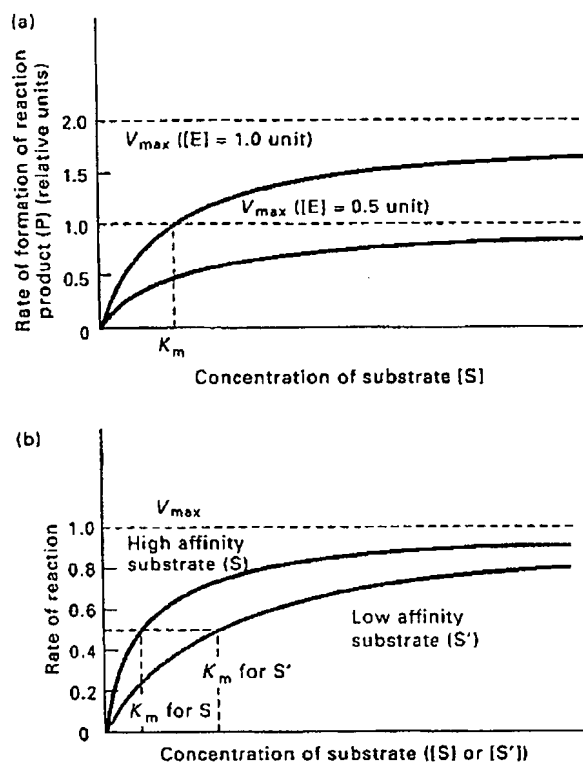
then

$$[ES] = \frac{[E]_{tot}}{1 + K_m/[S]}$$

Thus

$$\begin{aligned} \frac{d[P]}{dt} &= k_{cat} [ES] = k_{cat} [E]_{tot} \frac{1}{1 + K_m/[S]} \\ &= k_{cat} [E]_{tot} \frac{[S]}{[S] + K_m} \end{aligned} \quad (5)$$

This equation fits the curves shown in Figure 2-24a.  $V_{max}$ , which is equal to  $k_{cat} [E]_{tot}$ , is the maximal rate of product formation if all recognition sites on the enzyme are filled with substrate.  $K_m$  is equivalent to the substrate concentration at which the reaction rate is half-maximal. (If  $[S] = K_m$ , then from equation (5) we calculate the rate of product formation to be  $\frac{1}{2} k_{cat} [E]_{tot} = \frac{1}{2} V_{max}$ .) For most enzymes, the slowest step is the catalysis of [ES] to [E] + [P]. In these cases,  $k_{cat}$  is much less than  $k_2$ , so that  $K_m = (k_2 + k_{cat})/k_1 \approx k_2/k_1$ , is equal to the equilibrium constant for binding S to E. Thus the parameter  $K_m$  describes the affinity of an enzyme for its substrate. The smaller the value of  $K_m$ , the more avidly the enzyme can bind the substrate from a dilute solution (Figure 2-24b) and the lower the value of [S] needed to reach half-maximal velocity. The concentrations of the various



▲ **Figure 2-24** (a) The rate of a hypothetical enzymatically catalyzed reaction  $S \rightarrow P$  for two different concentrations of enzyme [E] as a function of the concentration of substrate [S]. The substrate concentration that yields a half-maximal reaction rate is denoted by  $K_m$ . Doubling the amount of enzyme causes a proportional increase in the rate of the reaction, so that the maximal velocity  $V_{max}$  is doubled. The  $K_m$ , however, is unaltered. (b) The rates of reactions catalyzed by an enzyme with a substrate S, for which the enzyme has a high affinity, and with a substrate S', for which the enzyme has a low affinity. The  $V_{max}$  value is the same for S and S', but  $K_m$  is higher for S'.

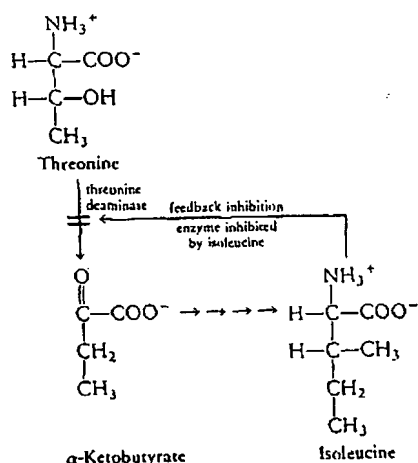
small molecules in a cell vary widely, as do the  $K_m$  values for the different enzymes that act on them. Generally, the intracellular concentration of a substrate is approximately the same as or greater than the  $K_m$  value of the enzyme to which it binds.

### The Actions of Most Enzymes Are Regulated

Many reactions in cells do not occur at a constant rate. Instead, the catalytic activity of the enzymes is *regulated* so that the amount of reaction product is just sufficient to meet the needs of the cell.



**An Enzyme Can Be Feedback Inhibited in a Reaction Pathway** Consider a series of reactions leading to the synthesis of the amino acid isoleucine, which is primarily used by cells as a monomer in the synthesis of proteins. The amount of isoleucine needed depends on the rate of protein synthesis in the cell. The first step in the synthesis of isoleucine is the elimination of an amino group, which converts the amino acid threonine to the compound  $\alpha$ -ketobutyrate. Threonine deaminase—the enzyme that catalyzes this reaction—plays a key role in regulating the level of isoleucine. In addition to its substrate-binding sites for threonine, threonine deaminase contains a binding site for isoleucine. When isoleucine is bound there, the enzyme molecule undergoes a conformational change, so that it cannot function as efficiently. Thus isoleucine acts as an *inhibitor* of the reaction for the conversion of threonine. If the isoleucine concentration in the cell is high, the binding of isoleucine to the enzyme temporarily reduces the rate of isoleucine synthesis:



This is an example of *feedback inhibition*, whereby an enzyme that catalyzes one of a series of reactions is inhibited by the ultimate product of the pathway.

In isoleucine synthesis, as in most cases of feedback inhibition, the final product in the reaction pathway inhibits the enzyme that catalyzes the first step that does not also lead to other products. The suppression of enzyme function is not permanent. If the concentration of free isoleucine is lowered, bound isoleucine dissociates from the enzyme, which then reverts to its active conformation. The binding of the inhibitor isoleucine to the enzyme and its subsequent release can be described by the equilibrium-binding constant  $K_i$ , which is similar to the constant  $K_m$  used for substrate binding:

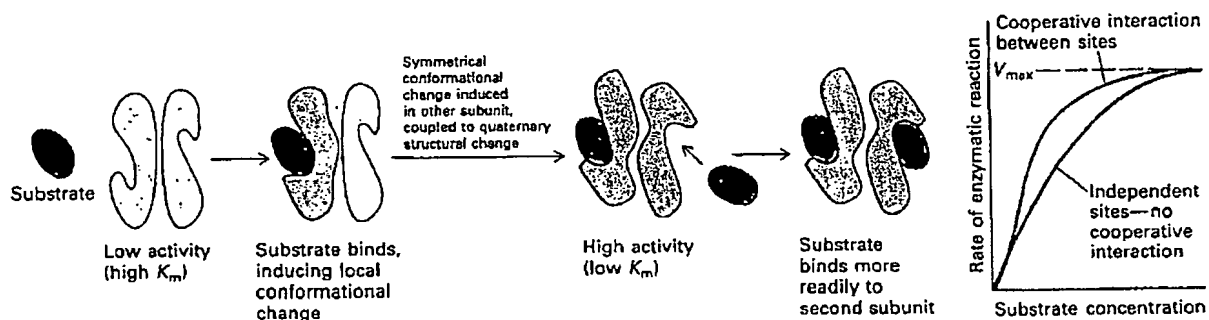
$$[\text{E} \cdot \text{Ile}]_{\text{inactive}} \xrightleftharpoons{K_i} [\text{Ile}] + [\text{E}]_{\text{active}}$$

$$K_i = \frac{[\text{Ile}][\text{E}]_{\text{active}}}{[\text{E} \cdot \text{Ile}]_{\text{inactive}}}$$

**Many Enzymes Have Multiple Binding Sites for Regulatory Molecules** Some enzymes have binding sites for small molecules that affect their catalytic activity; a stimulator molecule is called an *activator*. Enzymes may even have multiple sites for recognizing more than one activator or inhibitor. In a sense, enzymes are like microcomputers; they can detect concentrations of a variety of molecules and use that information to vary their own activities. Molecules that bind to enzymes and increase or decrease their activities are called *effectors*. Effectors can modify enzymatic activity because enzymes can assume both active and inactive conformations: activators are positive effectors; inhibitors are negative effectors. Effectors bind at *regulatory sites*, or *allosteric sites* (from the Greek for "another shape"), a term used to emphasize that the regulatory site is an element of the enzyme distinct from the catalytic site and to differentiate this form of regulation from competition between substrates and inhibitors at the catalytic site.

**Multimeric Organization Permits Cooperative Interactions among Subunits** Many enzymes and some other proteins are multimeric—that is, they contain several copies, or subunits, of one or more distinct polypeptide chains. Some multimeric enzymes contain identical subunits, each of which has a catalytic site and possibly an effector site. In other enzymes, regulatory sites and catalytic sites are located on different subunits, each with a particular structure. On binding an activator, inhibitor, or substrate, a subunit undergoes a conformational change, usually small, that triggers a change in quaternary structure. This quaternary rearrangement favors a similar conformational change in the other subunits, thereby increasing their affinity for the type of ligand initially bound (Figure 2-25). When several subunits interact cooperatively, a given increase or decrease in substrate or effector concentration causes a larger change in the rate of an enzymatic reaction than would occur if the subunits acted independently. Because of such *cooperative interactions*, a small change in the concentration of an effector or substrate can lead to large changes in catalytic activity.

Cooperative interactions among the four subunits in hemoglobin demonstrate clearly the advantages of multimeric organization. The binding of an  $\text{O}_2$  molecule to any one of the four chains (each hemoglobin chain binds one  $\text{O}_2$ ) induces a local conformational change in that subunit. This change can in turn induce a large change in quaternary structure. The quaternary change involves a rearrangement of the positions of the two  $\alpha$  and two  $\beta$  chains in the tetramer. The local conformational changes that accompany  $\text{O}_2$  binding can then occur more readily in the remaining subunits, increasing their affinity for oxygen. The binding of a second  $\text{O}_2$  makes the quaternary structural change even more likely. The cooperative

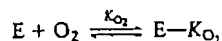


▲ **Figure 2-25** A cooperative interaction between active sites (two identical subunits of a hypothetical enzyme). The binding of a substrate to one subunit of a multimeric enzyme induces a conformational change in the adjacent subunit,

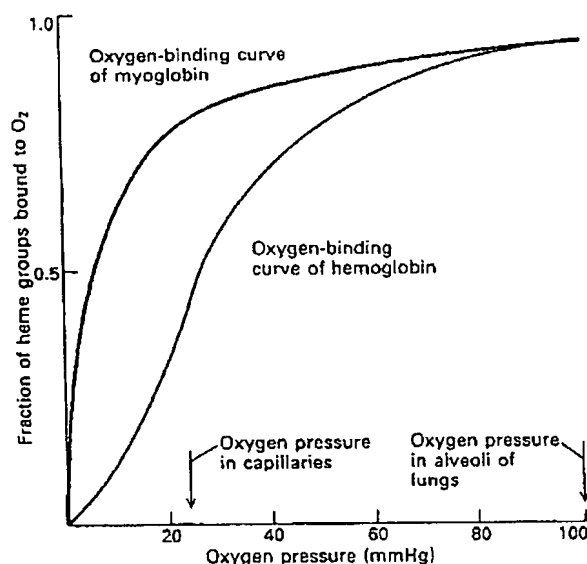
which lowers the  $K_m$  for the binding of the substrate there. Thus a small change in the substrate concentration can cause a much larger increase in the reaction rate than would occur if there were no cooperative interactions between active sites.

interaction between the chains causes the molecule to take up or lose four  $O_2$  molecules over a much narrower range of oxygen pressures than it would otherwise. As a result, hemoglobin is almost completely oxygenated at the oxygen pressure in the lungs and largely deoxygenated at the oxygen pressure in the tissue capillaries (Figure 2-26).

The contrast between hemoglobin and myoglobin is revealing. Myoglobin is a single-chain oxygen-binding protein found in muscle. The oxygen-binding curve of myoglobin has the characteristics of a simple equilibrium reaction:



Myoglobin has a greater binding affinity for  $O_2$  (a lower  $K_{O_2}$ ) than hemoglobin at all oxygen pressures. Thus, at



the oxygen pressure in capillaries,  $O_2$  moves from hemoglobin into the muscle cells, where it binds to myoglobin, ensuring the efficient transfer of  $O_2$  from blood to tissues.

The quaternary-structure rearrangements associated with multimeric organization also provide a way for the effects of activator or inhibitor binding at an allosteric site to be transmitted to a distant catalytic site without large changes in the secondary or tertiary structure of an enzyme, which would be incompatible with the principle that a particular primary structure must adopt a unique folded conformation. Thus, for example, small conformational changes in a domain in response to binding of an effector molecule would produce a quaternary-structure change, which amplifies the conformational signal and allows it to be transmitted robustly to other parts of the enzyme, where it would induce a small conformational change affecting enzymatic activity. Membrane-embedded receptor proteins that must transmit a conformational signal from one side of a membrane to the other are also likely to be multimeric; they transmit the signal by quaternary-structure rearrangement or by an effector-induced shift in the monomer-multimer equilibrium.

◀ **Figure 2-26** The binding of oxygen to hemoglobin depends on cooperative interactions between the four chains. The graph shows the fraction of heme groups in hemoglobin and in myoglobin bound to  $O_2$  as a function of the oxygen pressure. Note that the binding activity of hemoglobin increases sharply over a narrow range of oxygen pressures (20–40 mmHg). Hemoglobin is saturated with  $O_2$  in the lungs, but it releases much of its bound  $O_2$  at the low oxygen pressure in the tissue capillaries. At any oxygen pressure, myoglobin has a higher affinity for  $O_2$  than hemoglobin does. As myoglobin is a principal muscle protein, this property allows oxygen to be transferred from blood to muscle.

**Enzymes Are Regulated in Many Ways** The activities of enzymes are extensively regulated so that the numerous enzymes in a cell work together harmoniously. All metabolic pathways are closely controlled at all times. Synthetic reactions occur when the products of these reactions are needed; degradative reactions occur when molecules must be broken down. Kinetic controls affecting the activities of key enzymes determine which pathways are going to be used and the rates at which they will function.

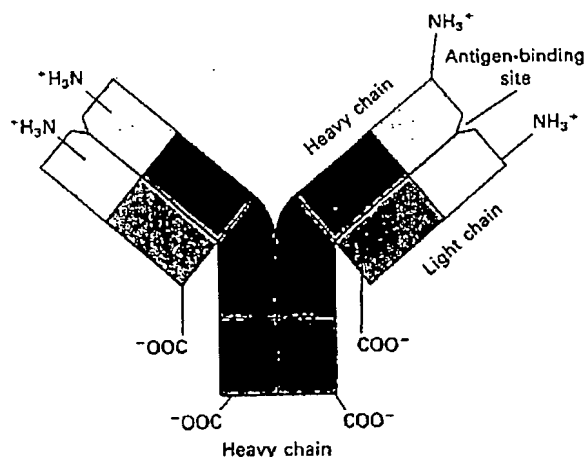
Regulation of cellular processes involves more than simply turning enzymes on and off, however. Some regulation is accomplished through *compartmentation*. Many enzymes are localized in specific compartments of the cell, such as the mitochondria or lysosomes, thereby restricting the substrates, effectors, and other enzymes with which an enzyme can interact. In addition, compartmentation permits reactions that might otherwise compete with one another in the same solution to occur simultaneously in different parts of a cell. Cellular processes are also regulated through the control of the rates of enzyme synthesis and destruction.

## Antibodies

Enzymes are not the only proteins that bind tightly and specifically to smaller compounds. The insulin receptor on the surface of a liver cell, for example, can bind to insulin so tightly that the receptors on a cell are half-saturated when the insulin concentration is only  $10^{-9}$  M. This protein does not bind to most other compounds present in blood; it mediates the specific actions of insulin on liver cells. A molecule other than an enzyme substrate that can bind specifically to a macromolecule is often called a *ligand* of that macromolecule.

The capacity of proteins to distinguish among different molecules is developed even more highly in blood proteins called *antibodies*, or *immunoglobulins*, than in enzymes. Animals produce antibodies in response to the invasion of an infectious agent, such as a bacterium or a virus. Antibodies will be discussed at length in Chapter 25. We introduce them here because they will appear as critical reagents in the discussions of many intervening chapters.

The recognition site of an antibody can bind tightly to very specific sites—generally on proteins or carbohydrates—on the surface of the infectious agent. Experimentally, animals produce antibodies in response to the injection of almost any foreign polymer; such antibodies bind specifically and tightly to the invading substance but, like enzymes, do not bind to dissimilar molecules. The antibody acts as a signal for the elimination of infectious agents. When it binds to a bacterium, virus, or virus-infected cell, certain white blood cells (leucocytes) recognize the invading body as foreign and respond by



▲ **Figure 2-27** The structure of an antibody molecule illustrated in an immunoglobulin (IgG) made of four polypeptide chains: two identical heavy chains (blue) and two identical light chains (orange). Each antigen-binding site is formed by the N-terminal segments of a heavy and a light chain. The N-termini are highly variable in sequence, giving rise to the wide range of antibody specificity.

destroying it. The specificity of antibodies is exquisite: they can distinguish between proteins that differ by only a single amino acid and between the cells of different individual members of the same species.

All vertebrates can produce a large variety of antibodies, including ones that bind to chemically synthesized molecules. Exposure to an antibody-producing agent, called an *antigen*, causes an organism to make a large quantity of different antibody proteins, each of which may bind to a slightly different region of the antigen. For a given antigen, these constellations of antibodies may differ from one member of a species to another.

Antibodies are formed from two types of polypeptides: heavy chains, each of which is folded into four domains, and light chains, each of which is folded into two domains (Figure 2-27). The N-terminal domains of both heavy and light chains are highly variable in sequence, giving rise to the specific binding characteristics of antibodies.

## Antibodies Can Distinguish among Closely Similar Molecules

The sequence of bovine insulin is identical to that of human insulin, except at three amino acids. Yet when bovine insulin is injected into people, some individuals respond by synthesizing antibodies that specifically recognize the specific amino acids in the bovine molecule, even though human beings generally do not produce anti-

# MICROBIOLOGY

## An Introduction

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*About the cover:* A technician is isolating plasmids, which are tiny circles of DNA found in bacteria. The plasmids are dissolved in a dye solution that fluoresces pink under ultraviolet light. Genetic engineering using plasmids is revolutionizing the biological sciences and industry (see pages 226-229 and 704-707).

Figure acknowledgments begin on page 749.

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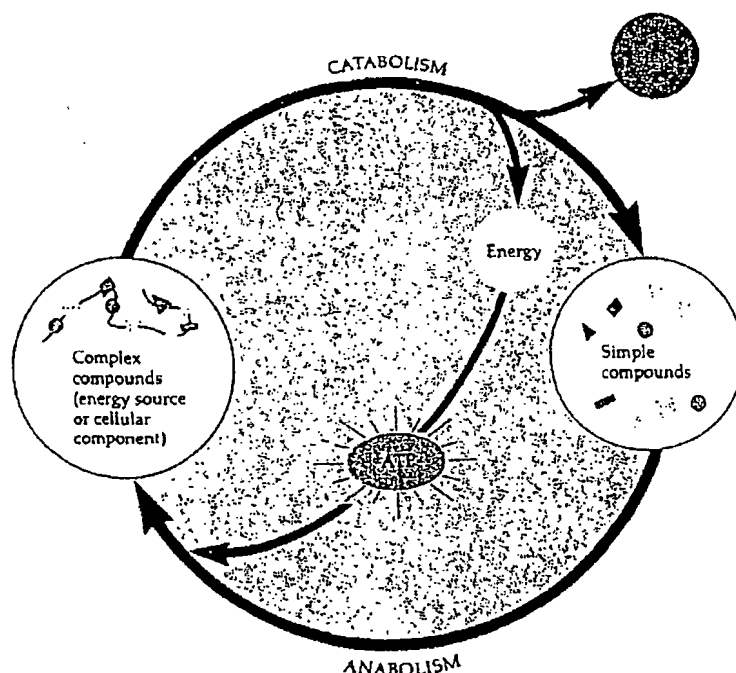
tial energy and therefore serve as energy carriers to drive energy-requiring reactions. The most common energy carrier in all biological systems is adenosine triphosphate (ATP); its structure can be reviewed in Figure 2-20. The role of ATP in the relationship between catabolic and anabolic processes is shown in Figure 5-1.

A little later in the chapter, we will examine some representative chemical reactions that deal with energy production (catabolic reactions) and energy utilization (anabolic reactions) in microorganisms. We will then look at how these various reactions are integrated within the cell. But first let us consider the principal properties of a group of proteins involved in almost all biologically important chemical reactions. These proteins, the enzymes, were described briefly in Chapter 2.

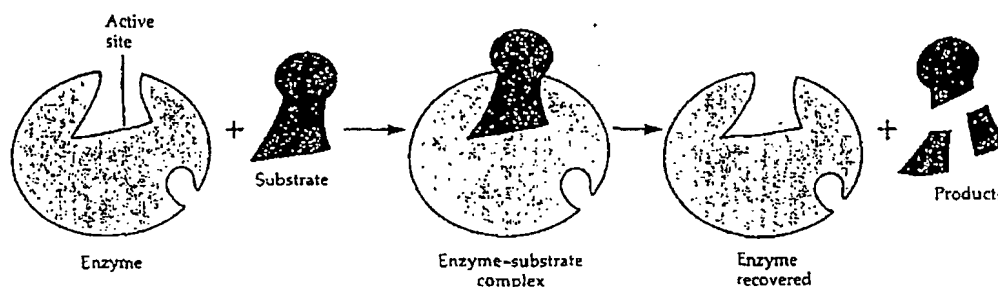
Although it is beyond the scope of this text to name and discuss the actions of individual enzymes, you should be aware of the central role of enzymes in metabolic reactions. It is important to understand that a cell's metabolic pathways are determined by its enzymes, which are, in turn, determined by its genetic makeup.

## ENZYMES

Many organic chemicals are so stable that they could remain unchanged in a cell for years. To activate these chemicals, living cells produce **enzymes**, proteins that act as catalysts in chemical reactions of importance to the cell. A *catalyst* is a substance that speeds up a reaction without being



**Figure 5-1** Relationship between anabolism and catabolism and the role of ATP. When simple compounds are combined to form complex compounds (anabolism), ATP provides the energy for synthesis. When large compounds are split apart (catabolism), heat energy is given off and some energy is trapped in ATP molecules.



**Figure 5-2** Mechanism of enzyme action. The surface of the substrate comes into contact with the active site on the surface of the enzyme to form an enzyme-substrate complex. The substrate is then transformed into products and the enzyme is recovered.

changed by it. Generally large globular proteins, enzymes range in molecular weight from about 10,000 to somewhere in the millions. Of the thousand or more known enzymes, each has a three-dimensional characteristic shape with a specific surface configuration due to its primary, secondary, and tertiary structures (see Figure 2-18).

### Mechanism of Enzyme Action

As mentioned in Chapter 2, catalysts lower the *activation energy* required for a chemical reaction. Although scientists do not completely understand how an enzyme does this, the sequence of events is believed to be as follows (Figure 5-2):

1. The surface of the *substrate*—that is, the molecule or molecules that are reactants in the chemical reaction to be catalyzed—contacts a specific region on the surface of the enzyme molecule, called the *active site*.
2. A temporary intermediate compound called an *enzyme-substrate complex* forms.
3. The substrate molecule is transformed (by rearrangement of existing atoms, a breakdown of the substrate molecule, or the combining of several substrate molecules).
4. The transformed substrate molecules, the products of the reaction, move away from the surface of the enzyme molecule.

5. The recovered enzyme, now freed, reacts with other substrate molecules.

Enzyme reaction is characterized by its extreme *specificity* for a particular substrate. For example, a specific enzyme may be capable of hydrolyzing a peptide bond only between two specific amino acids. And other enzymes are capable of hydrolyzing starch, but not cellulose; even though both starch and cellulose are polysaccharides composed of glucose subunits, the orientations of the subunits in the two polysaccharides differ. Enzyme specificity results from the three-dimensional shape of the active site, which fits the substrate somewhat like a lock with its key. In most instances, the substrate is much smaller than the enzyme, and relatively few of the enzyme's amino acids make up the active site.

A given compound can be a substrate for a number of different enzymes that catalyze different reactions. The fate of a given reactant (substrate) depends on the specific enzyme that reacts upon it. For example, glucose-6-phosphate, an important molecule in cell metabolism, may be acted upon by at least four different enzymes, each of which will give a different product.

Enzymes are exceedingly efficient. Under optimum conditions, they can catalyze reactions at rates that are  $10^8$  to  $10^{10}$  times (up to 10 billion times) more rapid than those of comparable reactions without enzymes. The *turnover number* (number of substrate molecules metabolized per enzyme mol-

Volume I

*Todd • Sanford • Davidsohn*

CLINICAL  
DIAGNOSIS *and*  
MANAGEMENT  
by  
LABORATORY  
METHODS

*Sixteenth Edition*

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destruction of the red cells with higher concentrations of the abnormal hemoglobin or selective removal of the abnormal hemoglobin from the cell.

In *heterozygous alpha hemoglobinopathies*, the abnormality in the alpha chain will affect all three hemoglobin types. Therefore, six different hemoglobin types are found—the three normal hemoglobins and the three abnormal forms. Examples are Hb D<sub>Baltimore</sub>, Hb Ann Arbor, and Hb M<sub>Boston</sub>.

Combinations of abnormalities exist. *Double heterozygotes for two beta chain abnormalities* produce two different abnormal beta chains; therefore, there are two abnormal hemoglobins and no hemoglobin A. An example of this is Hb S-C disease. Double heterozygotes for beta and delta chain abnormalities and for alpha and beta chain abnormalities are rare but have provided important information. The latter will have four major hemoglobin types on electrophoresis:  $\alpha_2^A\beta_2^A$ ;  $\alpha_2^X\beta_2^A$ ;  $\alpha_2^A\beta_2^Y$ ; and  $\alpha_2^X\beta_2^Y$ .

*Double heterozygotes for beta hemoglobinopathy and beta thalassemia* are well known. Here, the quantity of abnormal hemoglobin exceeds the normal hemoglobin, in contrast to the heterozygous beta hemoglobinopathies, in which the reverse is true. Examples are Hb S thalassemias and Hb E thalassemia.

#### Beta hemoglobinopathies

Hemoglobins S, C, D, and E are believed to be polymorphisms because their frequency is greater than can be explained by mutation alone (Lehmann, 1977). They occur in homozygous as well as heterozygous form and involve the beta chain.

**Sickle Cell Disease.** Homozygous Hb S disease is a serious chronic hemolytic anemia, first manifest in early childhood and often fatal before the age of 30 years. With modern medical care, however, many patients live longer. Hemoglobin S is found almost exclusively in the black population; 0.1 to 0.2 per cent of the blacks born in the United States have sickle cell anemia (Schneider, 1976).

In hemoglobin S the glutamic acid in the sixth position on the beta chain is replaced by valine. This substitution is on the surface of the molecule and changes its charge and, hence, its electrophoretic mobility. Hemoglobin S is freely soluble when fully oxygenated; when oxygen is removed from Hb S, polymerization of the abnormal hemoglobin occurs, forming tactoids (fluid crystals) which are

rigid and deform the cell into the shape which gave the cell its name (Fig. 29-7). In homozygous Hb S disease, sickling occurs at physiologic oxygen tensions and the rigidity of the red cells is responsible for the hemolysis as well as for most of the complications. The rigid cells are more vulnerable to trauma and are readily trapped by the reticuloendothelial system, especially the spleen, accounting for the hemolysis. As a result of the hemolysis, severe continued marrow hyperplasia during childhood produces bone changes: expansion of the marrow space, thinning of the cortex, and radial striations seen in the skull on x-ray. Leg ulcers are common.

**COMPLICATIONS.** In early childhood, bilateral painful swelling of the dorsa of the hands or feet occurs as a result of sickling and capillary stasis; this is known as the *hand-foot syndrome* or sickle cell dactylitis. It lasts about two weeks, is accompanied by changes of periostitis as observed by x-ray, and does not occur after the age of four.

The spleen is central to three complications: A *sequestration crisis* refers to sudden pooling of blood and rapid enlargement of the spleen, resulting in hypovolemic shock. This may occur in early childhood when splenomegaly is present. *Functional asplenia* (Pearson, 1969) consists of inadequate antibody responses under some conditions and an impaired ability of the reticuloendothelial system to clear bacteria and particulate material from the blood, probably due to reticuloendothelial blockade. This may partly explain the increased risk of infection in children with the disease. Salmonella and pneumococcal infections are unusually prevalent in children with sickle cell anemia. *Autosplenectomy* is the result of vaso-occlusive episodes, resulting in progressive infarction, fibrosis, and contraction of the spleen. Though splenomegaly is present in childhood, a small fibrotic remnant is the rule in the adult.

From early childhood, patients cannot produce a concentrated urine, apparently as a result of anoxic damage to the vasa recta in the medullae of the kidneys. Hematuria as a result of papillary necrosis is common.

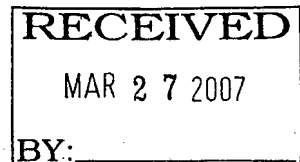
*Vaso-occlusive crises* are debilitating episodes of abdominal and bone or joint pain, accompanied by fever, which are probably due to plugging of small blood vessels by masses of sickled cells. Bone necrosis occurs and may be a focus for salmonella osteomyelitis. Aseptic necrosis of the femoral head is occasionally a complication. The various complications as a

The opinion in support of the decision being entered today is not binding precedent of the Board.

Paper 101

By: Trial Section Merits Panel  
Board of Patent Appeals and Interferences  
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Alexandria, VA 22313-1450  
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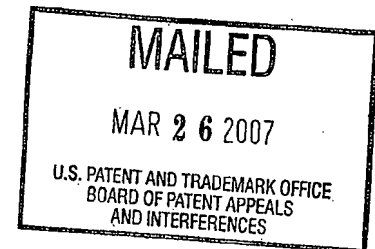
UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS  
AND INTERFERENCES  
(Administrative Patent Judge Richard E. Schafer)

**Human Genome Sciences, Inc.**  
Junior Party  
(Application 10/005,842-IFW  
Inventors: Jian Ni, Reiner L. Gentz,  
Guo-Liang Yu and Craig A. Rosen),

v.

**Immunex Corp.,**  
Senior Party  
(Patent 6,642,358  
Inventors: Charles Rauch and Henning Walczak)



Patent Interference No. 105,381 (RES)

Before: SCHAFER, HANLON and SPIEGEL, Administrative Patent Judges.  
SPIEGEL, Administrative Patent Judge.

**DECISION - MOTIONS - Bd.R. 125(a)**

**ADE- 38**  
USSN 10/052,798

1     **I.     Introduction**

2           This is a decision on the motions remaining in interference no. 105,381.

3           Junior party Ni has filed four motions. Senior party Rauch has filed five  
4     motions.

5           Ni substantive motion 1 to substitute Ni proposed count 2 for current  
6     Count 1 is **denied**. Ni substantive motion 2 for benefit for the purpose of priority  
7     is **dismissed** as moot as to Ni proposed count 2, **granted** as to the 29 July 1997  
8     filing date of the 60/054,021 application for Count 1 and otherwise **denied**. Ni  
9     substantive motion 3 seeking judgment that all Rauch's involved claims are  
10    unpatentable under 35 U.S.C. § 102(e) as anticipated by U.S. Patent 6,872,568  
11    is **denied**. Ni miscellaneous motion 4 to exclude certain evidence is **denied**.

12          Rauch substantive motion 1 for benefit for the purpose of priority as to  
13    Count 1 is **granted** as to the 28 March 1997 and 4 June 1997 filing dates of  
14    applications 08/829,536 and 08/869,852, respectively, and otherwise **denied**.  
15    Rauch substantive motion 2 to designate Ni claims 46, 55, 63, 64, 110 and 118  
16    as corresponding to Count 1 is **denied**. Rauch substantive motion 3 is **granted**  
17    to the extent that Ni claims 35, 36, 38-45, 47-54, 56-61, 75, 83, 92, 99, 100, 102-  
18    109, 111-116, 127-133, 168-178 and 180-203 are unpatentable under 35 U.S.C.  
19    § 102(e) as anticipated by U.S. Patent 6,072,047, **moot** as to anticipation under  
20    § 102(e) by U.S. Patents 6,642,358 and 6,569,642, and otherwise **denied**.  
21    Rauch responsive motion 4 is **dismissed** as moot in view of the denial of Ni  
22    substantive motion 1. Rauch miscellaneous motion 5 to exclude certain  
23    evidence is **dismissed** as moot.

**II. Findings of Fact (FF)**

The following findings of fact are supported by a preponderance of the evidence.

1. The junior party is Jian NI, Reiner L. GENTZ, Guo-Liang YU and Craig A. Rosen ("**NI**").
2. NI is involved in the interference on the basis of application 10/005,842 ("the '842 application," NX 2025), filed 7 December 2001.
3. The '842 application has been accorded benefit for the purpose of priority of the 17 March 1998 filing date of application 09/042,583 ("the '583 application," NX 2024).
4. NI's real party-in-interest is Human Genome Sciences, Inc. ("**HGS**").
5. The senior party is Charles RAUCH and Henning WALCZAK ("**Rauch**").
6. Rauch is involved in the interference on the basis of U.S. Patent 6,642,358 ("the '358 patent," RX 1012), issued 4 November 2005, based on application 09/578,392 ("the '392 application"), filed 25 May 2000.
7. The '392 application has been accorded benefit for the purpose of priority of the 26 June 1997 filing date of application 08/883,036 ("the '036 application," RX 1018), which issued 6 June 2000 as U.S. Patent 6,072,047 ("the '047 patent," RX 1048)
8. Rauch's real party-in-interest is Immunex Corp. ("**Immunex**").
9. The subject matter of the interference is defined by one count.
10. Count 1 is "Claim 6 of U.S. Patent 6,642,358" (Paper 1, p. 3).
11. Claim 6 of the '358 patent, written in independent form, reads:

1 A purified TRAIL-R polypeptide comprising an amino  
2 acid sequence that is at least 90% identical to the  
3 amino acid sequence presented in SEQ ID NO:2  
4 wherein said polypeptide binds TRAIL.

5 12. According to the '358 patent, SEQ ID NO:2 is the 440 amino acid  
6 sequence of a full length human receptor protein (including the N-  
7 terminal signal peptide), "TRAIL-R," encoded by the DNA of SEQ ID  
8 NO:1 (RX 1012, c. 1, l. 66 - c. 2, l. 2 and c. 22, ll. 7-11).

9 13. The claims of the parties are:

10	Ni	35-72, 75, 83, 92, 99-133, 152-178 and 180-203
11	Rauch	1-41

12 14. The claims of the parties which correspond to Count 1 are:

13	Ni	35, 36, 38-45, 47-54, 56-61, 75, 83, 92, 99, 100, 102-
14		109, 111-116, 127-133, 168-178 and 180-203
15	Rauch	1, 4-6, 8-11, 17-19, 26-28, 34, 37, 38 and 40

16 15. The claims of the parties which do not correspond to Count 1, and  
17 therefore are not part of this interference, are:

18	Ni	37, 46, 55, 62-72, 101, 110, 117-126 and 152-167
19	Rauch	2, 3, 7, 12-16, 20-25, 29-33, 35, 36, 39 and 41

20 Other findings of fact follow below.

21 **III. Ni Substantive Motion 1**

22 Pursuant to 37 CFR § 41.121(a)(1)(i), Ni moves to redefine the scope of  
23 the interference by substituting proposed count 2 for current Count 1 (Paper 29).  
24 Rauch opposes (Paper 52); Ni replies (Paper 60).

25 16. Ni's proposed count 2 reads (Paper 29, p. 1, ¶ 1):

26 A purified TRAIL-R polypeptide comprising an amino  
27 acid sequence that is at least 90% identical to the  
28 amino acid sequence presented in SEQ ID NO:2

1                    wherein said polypeptide binds TRAIL or induces  
2                    apoptosis.

3            17. According to Ni, its proposed count 2 simply incorporates Rauch claims 5  
4            and 6, as does the current count, and adds the language "or induces  
5            apoptosis" (*id.*).

6            It is our understanding that the source of SEQ ID NO:2 in Ni's proposed  
7            count 2 is the involved '358 patent of Rauch. With this understanding, we now  
8            address Ni motion 1.

9            Ni argues that the abilities to bind TRAIL and to induce apoptosis are  
10           inherent properties of the polypeptide of Count 1, although only the former is  
11           expressly recited in the count (Paper 29, p. 7, ¶ 3). A party seeking to change  
12           the count in an interference must demonstrate a genuine need to change the  
13           count. As stated in Louis v. Okada, 59 USPQ2d 1073, 1076 (Bd. Pat. App. & Int.  
14           2001),

15                    [a]t a minimum, ... a preliminary motion to  
16                    broaden out the count on the basis that a party's best  
17                    or earliest proofs are outside the current count (1)  
18                    should make a proffer of the party's best proofs, (2)  
19                    show that such best proofs indeed lie outside of the  
20                    scope of the current count, and (3) further show that  
21                    the proposed new count is not excessively broad with  
22                    respect to what a party needs for its best proofs.

23            Ni seeks to change the count by adding the limitation "or induces  
24            apoptosis" as an alternative to the limitation "binds TRAIL" (FF 16). Ni seeks to  
25            change the current count because its best proofs do not explicitly recite that the  
26            TRAIL-R polypeptide of the count binds TRAIL (FF 18). However, the fact that  
27            Ni's "best proofs" do not explicitly recite the language of the count does not alone

1 establish that those proofs are not directed to "subject matter" defined by the  
2 count. "The invention is not the language of the count but the subject matter  
3 thereby defined." Silvestri v. Grant, 496 F.2d 593, 598, 181 USPQ 706, 709  
4 (CCPA 1974). In appropriate circumstances, express limitations of the count  
5 may be shown to be inherent in the proofs, id. ("In reaching this conclusion, we  
6 do not disregard the fact that the count also requires that the ampicillin  
7 possesses greater storage-stability than hydrated ampicillin and have a  
8 molecular weight of about 349. However, we regard these as inherent properties  
9 of Form II ampicillin which add nothing to the count definition beyond that  
10 determined by the [other limitations].").<sup>1</sup> The limitation said not to be disclosed  
11 by Ni's best proofs, i.e., the ability to bind TRAIL, may be shown to be an  
12 inherent property of the TRAIL-R polypeptide of the count. In fact, Ni argues that  
13 the ability to bind TRAIL and the ability to induce apoptosis are both inherent  
14 properties of the TRAIL-R polypeptide of the count:

15           The ability to bind TRAIL is an expressly recited  
16           property of the polypeptide and it is an inherent  
17           property of the polypeptide of SEQ ID NO:2.  
18           Similarly, the ability of the polypeptide of SEQ ID  
19           NO:2 to induce apoptosis is also an inherent property  
20           of the polypeptide of SEQ ID NO:2.

21 [Paper 29, p. 7, ¶ 3 (citation to material facts omitted).] Additionally, Ni has not  
22 asserted that there are polypeptides meeting the amino acid sequence

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<sup>1</sup> In Silvestri, the count was directed to a new crystalline form of ampicillin which was "substantially free of water in the chemically bound state" and had a molecular weight of about 349, a particular infrared ("IR") spectrograph and improved storage stability vis-à-vis the previously known form of ampicillin. Id., 496 F.2d at 595-96, 181 USPQ at 709-710. The court held that it was sufficient to possess the claimed compound and to characterize it by its water content and IR spectrograph, without demonstrating the knowledge of the ampicillin's molecular weight because the molecular weight "add[s] nothing to the count beyond that determined by the water content and infrared spectrograph." Id., 496 F.2d at 599, 181 USPQ at 709.



1 requirements of the count which would induce apoptosis, but not bind TRAIL.  
2 Consequently, adding the phrase "or induces apoptosis" to Count 1 has not been  
3 shown to be necessary to encompass Ni's best proofs. Furthermore, changing  
4 the scope of the count would leave Ni in essentially the same position it is in now  
5 of having to prove an inherent property of the TRAIL-R polypeptide of the count  
6 (FF 18). Hence, Ni has failed to demonstrate that its best proofs are outside the  
7 scope of the current count and, therefore, that there is a genuine need to change  
8 the count.

9 Based on the foregoing, Ni substantive motion 1 is **denied**.

10 **IV. Rauch Responsive Motion 4**

11 Pursuant to 37 CFR § 41.121(a)(2), Rauch moves to be accorded benefit  
12 for the purpose of priority of the (i) 26 June 1997, (ii) 4 June 1997, (iii) 28 March  
13 1997, (iv) 12 March 1997 and (v) 13 February 1997 filing dates of U.S.  
14 applications (i) 08/883,036, (ii) 08/869,852, (iii) 08/829,536, (iv) 08/815,255 and  
15 (v) 08/799,861, respectively, as to Ni's proposed count 2 (Paper 45). Rauch  
16 responsive motion 4 is contingent upon the grant of Ni substantive motion 1 to  
17 substitute Ni's proposed count 2 for current Count 1. Since the contingency has  
18 not occurred, Rauch responsive motion 4 is **dismissed** as moot.

19 **V. Ni Substantive Motion 2**

20 Pursuant to 37 CFR §41.121(a)(1)(ii), Ni moves to be accorded benefit for  
21 the purpose of priority of the 17 March 1997 and 29 July 1997 filing dates of its  
22 earlier filed provisional applications 60/040,846 ("the '846 application," NX 2042)  
23 and 60/054,021 ("the '021 application," NX 2056), respectively, as to Count 1

1 and, contingent on the grant of Ni substantive motion 1, as to Ni's proposed  
2 count 2 (Paper 30). Rauch opposes (Paper 53); Ni replies (Paper 61).

3 To the extent Ni substantive motion 2 is contingent upon the grant of Ni  
4 substantive motion 1, it is **dismissed** as moot because the contingency has not  
5 occurred.

6 As discussed above, the subject matter of Count 1 is directed to a purified  
7 TRAIL-R polypeptide having an amino acid sequence that is at least 90%  
8 identical to SEQ ID NO:2 of Rauch's involved '358 patent, wherein the  
9 polypeptide binds TRAIL (FF 11).

10 18. TRAIL (TNF-Related Apoptosis-Inducing Ligand) is a member of the TNF  
11 ligand family known to be capable of inducing apoptosis when added to  
12 certain cells, e.g., Jurkat cells (NX 2096<sup>2</sup>).

13 19. The '021 and '846 application are both provisional applications.

14 20. The '021 application was filed 29 July 1997 (NX 2056, cover sheet).

15 21. The '846 application was filed 17 March 1997 (NX 2042, cover sheet).

16 22. Figure 1 of the '021 application is said to show the nucleotide and  
17 deduced amino acid sequences of "human Death Domain Containing  
18 Receptor 5" (DR5) obtained from the cDNA clone deposited as ATCC  
19 Deposit No. 97920 on 7 March 1997 (NX 2056, p. 1, ll. 7-9; p. 6, ll. 5-6; p.  
20 7, ll. 29-33; p. 9, ll. 9-12; p. 10, ll. 34-35).

21 23. According to the '021 specification, DR5 is a 411 amino acid protein (id.,  
22 p. 26, ll. 9-10).

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<sup>2</sup> Wiley et al., "Identification and Characterization of a New Member of the TNF Family that Induces Apoptosis," Immunity, Vol. 3, pp. 673-682 (December 1995) (NX 2096).

- 1       24. Example 6 of the '021 specification is said to show that a DR5  
2       extracellular domain-Fc fusion construct (DR5-Fc) binds TRAIL (id., p.  
3       50, l. 6 - p. 51, l. 2; Figures 6A-6C).
- 4       25. Figure 1 of the '846 application is said to show the nucleotide and  
5       deduced amino acid sequences of DR5 obtained from the cDNA clone  
6       deposited as ATCC Deposit No. 97920 on 7 March 1997 (NX 2042, p. 1,  
7       ll. 5-6; p. 3, ll. 22-25; p. 5, ll. 24-27).
- 8       26. According to the '846 specification, DR5 is a 411 amino acid protein (id.,  
9       p. 6, ll. 25-27).
- 10      27. Figure 2 of the '846 application is said to compare the deduced amino  
11      acid sequence of DR5 to the amino acid sequences of three known TNF  
12      family death receptor proteins -- human tumor necrosis factor receptor 1  
13      (human TNFR1), human Fas protein and DR3 protein (id., p. 5, ll. 8-13).
- 14      28. According to the '846 specification, similarities between the amino acid  
15      sequences shown in Figure 2 "**strongly suggest** that DR5 is also a  
16      death domain containing receptor with the ability to induce apoptosis,"  
17      i.e., that DR5 is a putative death receptor protein of the TNF receptor  
18      family (id., p. 6, ll. 31-33, emphasis added).
- 19      29. Further according to the '846 specification, "TNF-family ligands induce  
20      various cellular responses by binding to TNF-family receptors, including  
21      the DR5 of the present invention. Cell which express the DR5  
22      polypeptide **are believed to have** a potent cellular response to DR5  
23      ligands ..." (id., p. 26, ll. 12-15, emphasis added).

- 1       30. The '846 specification defines "TNF-family ligand" as  
2               naturally occurring, recombinant, and synthetic  
3               ligands that are capable of binding to a member of the  
4               TNF-receptor family and inducing the ligand/receptor  
5               signaling pathway. Members of the TNF ligand family  
6               include, but are not limited to, **DR5 ligands**, TRAIL,  
7               TNF- $\alpha$ , lymphdotoxin- $\alpha$  (LT- $\alpha$ , also known as TNF- $\beta$ ),  
8               LT- $\beta$  (found in complex heterotrimer LT- $\alpha$ 2- $\beta$ ), FasL,  
9               CD40, CD27, CD30, 4-1BB, OX40 and nerve growth  
10              factor (NGF). [Id., p. 31, ll. 4-9, emphasis added.]
- 11       31. The amino acid sequence of the DR5 protein shown in the respective  
12              Figures 1 of the '021 and '846 applications are identical.
- 13       32. It is undisputed that the amino acid sequences shown in Figures 1 of the  
14              '021 and '846 applications are at least about 93% identical to the amino  
15              acid sequence of SEQ ID NO:2 as recited in Count 1, with 411 of 440  
16              total amino acids being identical (see Paper 53, p. 22 where Rauch  
17              admits Ni's Statement of Material Facts (SMFs) 7 and 8 as set forth in  
18              Paper 30, p. 26).
- 19       33. Thus, the '021 application describes an enabled embodiment within the  
20              scope of Count 1, i.e., a DR5 polypeptide having an amino acid  
21              sequence that is at least 90% identical to the amino acid sequence of  
22              SEQ ID NO:2 of the '358 patent (FFs 22, 31 and 32) and which binds  
23              TRAIL (FF 24).
- 24       34. Rauch does not dispute Ni's claim to benefit for the purpose of priority of  
25              the filing date of the '021 application (Paper 53).
- 26       Based on the foregoing, we accord Ni benefit for the purpose of priority of  
27       the filing date of the '021 application as to Count 1.

1        While the '846 application describes (Figure 1) a DR5 polypeptide having a  
2        deduced amino acid sequence which is at least 90% identical to the amino acid  
3        sequence set forth in SEQ ID NO:2 of the '358 patent (FF 32), the disclosure of  
4        the '846 application suggests that the DR5 polypeptide is a death domain  
5        containing receptor with the ability to induce apoptosis (FF 28). However, the  
6        disclosure of the '846 application does not describe preparing a DR5 polypeptide  
7        (or ligand binding portion thereof) or binding the ligand TRAIL to the DR5  
8        polypeptide (or ligand binding portion thereof). Rather, the disclosure of the '846  
9        application suggests that a DR5 polypeptide binds a "DR5 ligand" (FFs 29 and  
10       30).

11       Ni's position is premised on classifying DR5 as a "putative TNF death  
12       receptor" based on the described similarity between the amino acid sequences of  
13       DR5 and three previously known TNF death receptors TNFR1, Fas and DR3 in  
14       the '846 application. According to Ni, TNFR1, Fas and DR3 were all known to  
15       induce apoptosis upon activation and, therefore, that same function should be  
16       imputed to DR5 by virtue of the described similarity in amino acid sequences  
17       between DR5 and the three TNF death receptors. Ni argues that the '846  
18       specification explicitly teaches that DR5 induces apoptosis and binds to a TNF  
19       ligand selected from a limited list including TRAIL. Ni further argues that, based  
20       on the doctrine of inherency, the '846 application need not expressly recite that  
21       DR5 binds TRAIL so long as the '846 application describes the subject matter of  
22       the Count. [Paper 30, p. 2, ¶ 3 and ¶ bridging pp. 9-10.]

- 1       35. Ni relies on the direct testimony of John C. Reed, M.D., Ph.D. (NX 2103)
- 2       in support of its position.
- 3       36. Dr. Reed has been qualified as an expert to give opinions on the subjects
- 4       of apoptosis and of the tumor necrosis family of ligands (TNFs) and
- 5       receptors (TNFRs), including death receptors.
- 6       37. According to Dr. Reed, the deduced amino acid sequence of human DR5
- 7       described in the '846 application has all the canonical (structural)
- 8       features of a classic death receptor of the TNFR family, i.e., a leader
- 9       peptide, conserved cysteine-rich domain(s), a transmembrane domain
- 10      and a cytosolic domain containing a "death domain" (NX 2103, ¶ 28).
- 11      38. Further according to Dr. Reed, the death domain "is necessary and
- 12      sufficient for apoptosis induction, at least when overexpressed in
- 13      mammalian cells" (*id.*, ¶ 21).
- 14      39. Still further according to Dr. Reed, DR5 shares the highest degree of
- 15      amino acid sequence identity with then known death receptor proteins
- 16      human TNFR1, Fas and DR3 (*id.*, ¶ 29).
- 17      40. Dr. Reed states that the deduced amino acid sequence of the "death
- 18      domain" region of the DR5 protein described in Ni's '846 application was
- 19      approximately 21, 32 and 33 percent identical to the amino acid
- 20      sequences of the death domains of Fas, TNFR1 and DR3, respectively,
- 21      "using Lipman-Pearson Protein Alignment (with the following parameters:
- 22      Ktuple 2; Gap Penalty 4; Gap Length Penalty 12)" (*id.*, ¶ 31).

- 1       41. Dr. Reed opines that a death domain amino acid sequence identity of
- 2       approximately 21-33 percent is "significant" because Chinnaniyan (NX
- 3       2058) reported that the death domain of DR3 was 47 and 23 percent
- 4       identical to that of TNFR1 and Fas, respectively, while Marsters (NX
- 5       2059) reported that the death domain of DR3 was 48 and 20 percent
- 6       identical to that of TNFR1 and Fas, respectively (NX 2103, ¶ 31).
- 7       42. Chinnaiyan reported using MegAlign™ software to align the compared
- 8       amino acid sequences (NX 2058, Fig. 1).
- 9       43. MegAlign™ software can create alignments between two or more
- 10      sequences according to different methods, e.g., the clustal method or the
- 11      Jotun Hein method (see e.g., U.S. Patent 6,277,568, col. 8, ll. 22-41).
- 12      44. Neither Chinnayian or Marsters reported the alignment program and
- 13      parameters used to obtain their respective percent sequence identity
- 14      scores.
- 15      45. Dr. Reed did not explain percent sequence identity scoring, e.g., how
- 16      different alignment methods and parameters calculate percent sequence
- 17      identity scores; how different alignment methods are compared
- 18      (normalized to account for the use of different parameters, e.g.,
- 19      sequence lengths, gaps, gap positions, etc.); the significance, if any, of
- 20      comparing sequences within predicted structural features (e.g., a death
- 21      domain or extracellular domain) versus over the entire primary amino
- 22      acid sequence; standard error of the method(s) used; use of iteration,
- 23      etc.

1 46. For example, according to Tartaglia,<sup>3</sup>

2 [i]t has been noted previously that the intracellular  
3 domain of TNF-R1 shares a **weak homology (29%**  
4 **identity over 45 amino acids)** with the intracellular  
5 domain of Fas antigen. Upon further inspection of  
6 these sequences, we noted that introduction of a 1  
7 amino acid gap in the Fas sequence extended the  
8 region of homology an additional 20 amino acids  
9 (Figure 3). [NX 2067, p. 846, col. 2, ¶ 1, emphasis  
10 added.]

11 47. Nonetheless, Dr. Reed believes that one of ordinary skill in the art would  
12 have reasonably expected the putative death receptor DR5 of the '846  
13 specification to have utilities similar to the known utilities of known death  
14 receptors TNFR1, Fas and DR3 (NX 2103, ¶¶ 33-34).

15 48. According to Dr. Reed, "**the most reasonable conclusion to draw from**  
16 Ni's March 17, 1997 application is that DR5 is expected, by persons of  
17 ordinary skill in the art, to be a novel death receptor" and, therefore,  
18 skilled artisans "**would have predicted** that activation of DR5 would  
19 induce apoptosis" (NX 2103, ¶ 32, emphasis added).

20 49. Further according to Dr. Reed, activation (aggregation) of a death  
21 receptor could be caused by (i) ligand binding to the death receptor, (ii)  
22 antibody binding to the death receptor or (iii) overexpression of the death  
23 receptor on the cell surface (*id.*, ¶ 24).

24 50. Dr. Reed testified that  
25 if one would want to determine which TNF ligand DR5  
26 binds, Ni's March 17, 1997 application [i.e., the '846  
27 application], in combination with what was known in  
28 the art at the time, provides all of the necessary  
29 information. For example, Ni's March 17, 1997

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<sup>3</sup> Tartaglia et al. (Tartaglia), "A Novel Domain within the 55 kd TNF Receptor Signals Cell Death,"  
*Cell*, Vol. 74, pp. 845-853 (10 September 1993) (NX 2067).



1 application states that **DR5 binds to a TNF-family**  
2 **ligand** (Exhibit 2042, pg. 4, ¶¶2-3; pg. 26, ¶1; pgs 28-  
3 29; pg. 31, ¶1, pg. 31, ¶1 [sic]), which would have  
4 been expected by a person of ordinary skill in the art  
5 in view of the literature that was available by March  
6 17, 1997. Additionally, Ni's March 17, 1997  
7 application specifically defines "a TNF family ligand"  
8 as a limited number of molecules, one of which is  
9 TRAIL. (Exhibit 2042, pg. 31, lines 4-9). The Ni  
10 March 17, 1997 application also teaches assays, such  
11 as cellular response **assays, that could be used to**  
12 **determine whether TRAIL, or any other of the listed**  
13 **TNF ligands, binds to DR5.** (Exhibit 2042, pg. 26,  
14 lines 12-26; pg. 27, line 21 through pg. 29, line 6).  
15 **Alternatively, as of March 17, 1997, it would have**  
16 **been routine for a person of ordinary skill in the art to**  
17 **have tested whether DR5 binds to the TNF-family**  
18 **ligands recited in Ni's May [sic] 17, 1997**  
19 **application, including TRAIL.** Thus, if one wanted to  
20 have determined whether DR5 bound to a TNF  
21 ligand, including TRAIL, the Ni March 17, 1997  
22 application, in combination with what was known in  
23 the art at the time, teaches all of the needed  
24 information. [NX 2103, ¶ 56, emphasis and bracketed  
25 text added.]

26 51. Dr. Reed notes that while most TNF family receptors have been shown  
27 experimentally to bind to specific TNF family ligands, some receptors "do  
28 not have known receptors to date, or a delay of many years occurred  
29 before the specific ligand was established" (NX 2103, ¶ 18).

30 52. According to the '846 specification, there are eleven known members of  
31 the TNF ligand family, i.e., TNF- $\alpha$ , lymphotoxin- $\alpha$  (LT- $\alpha$ , also known as  
32 TNF- $\beta$ ), LT- $\beta$  (found in complex heterotrimer LT- $\alpha$ 2- $\beta$ ), FasL, CD40,  
33 CD27, CD30, 4-1BB, OXO40, nerve growth factor (NGF) and TRAIL (NX  
34 2042, p. 1, ll. 21-25 and p. 31, ll. 6-9).

35 53. The '846 specification defines "TNF-family ligand" as

1 naturally occurring, recombinant, and synthetic  
2 ligands that are capable of binding to a member of the  
3 TNF receptor family and inducing the ligand/receptor  
4 signaling pathway. Members of the TNF ligand family  
5 include, but are not limited to, **DR5 ligands**, TRAIL,  
6 TNF- $\alpha$ , lymphdotoxin- $\alpha$  (LT- $\alpha$ , also known as TNF- $\beta$ ),  
7 LT- $\beta$  (found in complex heterotrimer LT- $\alpha$ 2- $\beta$ ), FasL,  
8 CD40, CD 27, CD30, 4-1BB, OX40 and nerve growth  
9 factor (NGF). [*Id.*, p. 31, ll. 4-9, emphasis added.]

10 54. Dr. Reed relies on Ni's later filed '201 application (NX 2056, Figure 6A)  
11 and on a later published August 1997 article (NX 2031<sup>4</sup>) to support his  
12 testimony that DR5 "necessarily" binds to TRAIL and "necessarily"  
13 induces apoptosis (NX 2103, ¶ 57).

14 To be accorded benefit for the purpose of priority in an interference  
15 proceeding "means Board recognition that a patent application provides a proper  
16 constructive reduction to practice under 35 U.S.C. 102(g)(1)." 37 CFR § 41.201.  
17 A constructive reduction to practice "means a described and enabled anticipation  
18 under 35 U.S.C. 102(g)(1) in a patent application of the subject matter of a  
19 count." *Id.* To fulfill the written description requirement, the patent specification  
20 must describe an invention in sufficient detail that one skilled in the art can  
21 clearly conclude that the inventor invented what is claimed. Lockwood v. Am.  
22 Airlines, Inc., 107 F.3d 1565, 1572, 41 USPQ2d 1961, 1966 (Fed. Cir. 1997).  
23 The specification "need not describe the claimed subject matter in exactly the  
24 same terms as used in the claims; it must simply indicate to persons skilled in the  
25 art that as of the [filing] date the applicant had invented what is now claimed."  
26 Eiselstein v. Frank, 52 F.3d 1035, 1038, 34 USPQ2d 1467, 1470 (Fed. Cir. 1995)

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<sup>4</sup> Guohua et al. (Guohua), "An Antagonist Decoy Receptor and a Death Domain-Containing Receptor for TRAIL," Science, Vol. 277, pp. 815-818 (8 August 1997). Three of the six coauthors are also Ni inventors.

1 (citations omitted). Furthermore, "the fact that a characteristic is a necessary  
2 feature or result of a prior-art embodiment (that is itself sufficiently described and  
3 enabled) is enough for inherent anticipation, even if that fact was unknown at the  
4 time of the prior invention." Toro Co. v. Deere & Co., 69 USPQ2d 1584, 1590  
5 (Fed. Cir. 2004) (citations omitted). Benefit for the purpose of priority focuses on  
6 the subject matter of a count and only requires a constructive reduction to  
7 practice of a single embodiment within the scope of the count. Falkner v. Inglis,  
8 463 F.3d 1376, 1379, 79 USPQ2d 1001, 1004 (Fed. Cir. 2006); Hunt v.  
9 Treppschuh, 523 F.2d 1386, 1389, 187 USPQ 426, 429 (CCPA 1975).<sup>5</sup>

10 Here, the subject matter of the count is directed to a functional protein, i.e., a  
11 purified TRAIL-R polypeptide having an amino acid sequence that is at least 90%  
12 identical to SEQ ID NO:2 of Rauch's involved '358 patent, wherein the  
13 polypeptide binds TRAIL (FF 11). Relying on the testimony of Dr. Reed, Ni  
14 argues that the similarity between the deduced amino acid sequence of DR5 and  
15 the known amino acid sequences of three TNF death receptor proteins, i.e.,  
16 TNFR1, Fas and DR3, as described in the '846 application is sufficient to  
17 characterize DR5 as a putative TNF death receptor protein and to predict that  
18 DR5 has utilities/functions similar to those of known death receptor proteins, e.g.,  
19 induction of apoptosis upon activation.

20 Neither the disclosure of the '846 application nor the testimony of Dr. Reed  
21 is as explicit as Ni argues. The '846 application suggests that DR5 is a putative  
22 TNF death receptor protein (FF 28). Dr. Reed testified that the most reasonable

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<sup>5</sup> In contrast, benefit for the purpose of 35 U.S.C. § 120 and related statutes focuses on the subject matter of the claim and requires the application for which benefit is sought to describe and enable the entire scope of the claim.

1 conclusion a person of ordinary skill in the art would draw from the '846  
2 application is that DR5 "is expected ... to be a novel death receptor" (FF 48).  
3 The '846 specification does not describe preparing DR5 or a ligand binding  
4 portion thereof (e.g., expressing and purifying DR5 from the DNA of Figure 1).  
5 The '846 specification does not describe an activated (functional) DR5 or identify  
6 the TNF ligand which activates (binds to) DR5.

7       Since TRAIL was known to be capable of inducing apoptosis (FF 18),  
8 identifying TRAIL as the TNF ligand which bound to DR5 in the '846 specification  
9 would have been one way of describing DR5 as capable of inducing apoptosis.  
10 Dr. Reed testified that '846 application "states that DR5 binds to a TNF-family  
11 ligand" and that there were "assays, that could be used to determine whether  
12 TRAIL, or any other of the listed TNF ligands, binds to DR5" (FF 50). Dr. Reed  
13 further testified that "it would have been routine for one of ordinary skill in the art  
14 to have tested whether DR5 binds to the TNF-family ligands recited" in the '846  
15 application, "including TRAIL" (FF 50). Notably, the '846 specification  
16 enumerates "DR5 ligands" as separate and distinct ligands in its list of TNF  
17 ligands, including TRAIL (FF 53), implying that DR5 might bind to either a known  
18 TNF ligand, e.g., TRAIL, or an as yet unknown TNF ligand, i.e., a DR5 ligand, or  
19 another TNF ligand known to be capable of inducing another function, e.g., cell  
20 proliferation.

21       In short, there is neither explicit nor implicit disclosure in the '846  
22 application said to show that the DR5 polypeptide encoded by the DNA of Figure  
23 1 is a functional/bioactive protein. The cognate ligand for DR5 is not explicitly

1 identified in the '846 application, although it would have been routine for one of  
2 ordinary skill in the art to do so using known techniques, as testified to by Dr.  
3 Reed (FF 50). Moreover, there could be no explicit description of an activated  
4 DR5 based on antibody binding or overexpression in mammalian cells absent  
5 obtaining the DR5 polypeptide (e.g., by expressing the product of the DNA of  
6 Figure 1) against which to raise an antibody. Finally, a person skilled in the art  
7 could not have reasonably predicted the function(s) of DR5 based solely on the  
8 similarity between its deduced amino acid sequence as set forth in Figure 1 of  
9 the '846 application and the known amino acid sequences of TNFR1, Fas and  
10 DR3 in view of the state of the art when the '846 application was filed for the  
11 following reasons.

12 Genes encode proteins by providing a sequence of nucleic acids that is  
13 translated into a sequence of amino acids. Methods used to identify novel genes  
14 are classified into two types, i.e., homology based or non-homology based. In  
15 homology based methods, for example, clones from a cDNA library are cloned  
16 and analyzed (sequenced). The resultant nucleotide sequences and/or deduced  
17 amino acid sequences are checked against databases for similarity (homology)  
18 to previously characterized sequences on the theory that molecules with similar  
19 sequences would be expected to perform similar functions. However, one of the  
20 difficulties in identifying a functional protein is that function depends not only on  
21 the amino acid sequence of the protein, but also on other factors, e.g., the three-  
22 dimensional structure of the protein.

1           In order for a protein to function properly its amino acid sequence (primary  
2 structure) must fold itself up into a complex three-dimensional shape which  
3 allows for molecular recognition. Molecular recognition often involves only a  
4 small number of key amino acid residues on the functional surfaces of interacting  
5 molecules. These residues are dispersed in diverse regions of the primary  
6 amino acid sequence due to the complex structural organization of the protein.  
7 There are multiple levels to the structural organization of a protein. The *primary*  
8 *structure* of a protein refers to the linear arrangement of amino acid residues  
9 along a polypeptide chain. *Secondary structures* form through interactions  
10 between amino acids typically found near each other in the peptide chain which  
11 fold parts of the chain into regular structures, e.g.,  $\alpha$  helices and  $\beta$  sheets.  
12 *Tertiary structure* folds both the secondary structures and the regions between  
13 them into compact three-dimensional shapes in an energetically favourable way.  
14 *Quaternary structure* refers to the organization of several polypeptide chains into  
15 a single protein molecule, e.g., hemoglobin is a tetramer. Consequently, amino  
16 acid residues rather near to each other in a protein's primary structure may be  
17 rather distant in the protein's ultimate quaternary structure. [See generally,  
18 MOLECULAR CELL BIOLOGY ("MCB"), second edition, Darnell et al., W.H.  
19 Freeman and Company, New York, NY (1990), pp. 44-48 (copy enclosed).]  
20           For example, an enzyme is a protein that catalyzes a biochemical  
21 reaction. The function of an enzyme relies on the structure of its "active site," a  
22 specific cavity-like region on the surface of the three-dimensional enzyme which  
23 allows a spatial fit (molecular recognition) between the enzyme and its substrate

1 (reactant in the reaction being catalyzed). The active site contains key amino  
2 acids that bind the substrate and are involved in the reaction catalyzed by the  
3 enzyme. These key amino acids are brought into proximity (into the active site)  
4 by protein folding. [See generally, MICROBIOLOGY: An Introduction, Tortora et  
5 al., The Benjamin/Cummings Publishing Company, Inc., Menlo Park, California  
6 (1982), pp. 111-112, copy enclosed; MCB, pp. 55-65, copy enclosed.]

7 On the other hand, mutations that cause human disease often disrupt  
8 protein structure, thereby altering or abolishing normal protein function. For  
9 example, sickle cell anemia occurs in humans that are homozygous for a  $\beta$ -  
10 hemoglobin gene that differs from the normal adult hemoglobin gene by a single  
11 base pair, resulting in a change in a single amino acid from glutamate to valine in  
12 position 5. This substitution is on the surface of the abnormal hemoglobin (Hb S)  
13 and changes the electrostatic charge on the surface of Hb S. When oxygen is  
14 removed from Hb S, the protein polymerizes into rigid crystals that deform a  
15 sickle cell patient's red blood cells. Thus, although normal hemoglobin and Hb S  
16 have virtually identical primary amino acid sequences, a single amino acid  
17 change in Hb S alters its quaternary structure and results in abnormal protein  
18 function. [See generally, CLINICAL DIAGNOSIS AND MANAGEMENT BY  
19 LABORATORY METHODS, sixteenth edition, J.B. Henry ed., W.B. Saunders  
20 Company, Philadelphia (1979), Vol. I, p. 992, copy enclosed.]

21 Therefore, "[s]equence comparison can indicate whether an RNA or  
22 protein molecule or region of DNA is already known (identity) or has some  
23 degree of similarity to a known sequence" (MOLECULAR BIOLOGY AND

1 BIOTECHNOLOGY, R. Myers, ed., VCH Publishers, Inc., New York, NY (1995),  
2 p. 860, c. 1, ¶ 1, copy enclosed). However, since "[t]he function of nucleic acids  
3 and proteins depend on their structure and involves complex interactions in three  
4 dimensions",

5 [i]t is not presently understood whether it is possible,  
6 in general, to derive structure from sequence.  
7 Sequence alone is therefore often inadequate to  
8 determine function. Predictions made from sequence  
9 analysis need to be experimentally tested.  
10 Nonetheless, computer analysis of sequences is  
11 valuable in suggesting the most useful experiments to  
12 perform. [*Id.*, p. 860, c. 1, ¶ 2.]

13 Indeed, the difficulties in predicting the structure and function of a protein from  
14 just its amino acid sequence (primary structure) are so well known in the art that  
15 the ability to characterize the function and structure of a protein from its amino  
16 acid sequence has been called the "Holy Grail" of molecular biology (RX 1061,<sup>6</sup>  
17 p. 511, c. 2, ¶ 1 to p. 512, c. 1, ¶ 1).

18 55. Genchong Cheng, Ph.D., is a witness for Rauch and has been qualified  
19 as an expert to give opinions on the subjects of signal transduction and  
20 gene expression networks through the TNFR, Toll-like receptor (TLR)  
21 and Nod receptor families during immune responses.

22 56. Dr. Cheng testified that  
23 [s]equence homology to other death domain-  
24 containing TNF receptors may be sufficient to  
25 convince one of ordinary skill in the art that a novel  
26 protein is a TNFR family member. However,  
27 sequence homology alone is not sufficient to support  
28 an assertion that a novel TNFR family member  
29 protein will induce specific biological activities such as

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<sup>6</sup> Pawlowski et al., "From fold to function predictions: an apoptosis regulator protein BID,"  
Computers and Chemistry, Vol. 24, pp. 511-517 (2000) (RX 1061).



1 apoptosis. Without additional data regarding the  
2 activity of a TNFR family member, such as, for  
3 example, the identity of the ligand with a known  
4 function (such as TRAIL) to which the receptor binds,  
5 one of ordinary skill in the art cannot reasonably  
6 predict the function of the TNFR family member. [RX  
7 1039, ¶ 17.]

8 Ni's own witness, Dr. Reed, did not testify that the specification and figures  
9 of the '846 application would have reasonably conveyed to a skilled artisan that a  
10 DR5 having the deduced amino acid sequence shown in Figure 1 is in fact a  
11 functional death receptor protein based solely on its amino acid sequence  
12 (primary structure). Dr. Reed did not testify that the skilled artisan would have  
13 understood the '846 application to describe a functional death receptor. Rather,  
14 Dr. Reed testified to "the most reasonable" (not the necessary and always)  
15 conclusion that one of ordinary skill in the art would have drawn from the  
16 disclosure of the '846 application (FF 48).

17 Dr. Reed also testified that there was a "significant" percent sequence  
18 identity between the deduced amino acid sequence of DR5's death domain and  
19 the amino acid sequence of the death domains of TNFR1, Fas and DR3 (FFs 40  
20 and 41). However, Dr. Reed's testimony in this regard is entitled to little, if any,  
21 weight because Dr. Reed did not provide a sufficient basis for his opinion. Dr.  
22 Reed did not explain how percent sequence identity scores were obtained,  
23 identify what alignment methods and parameters were used by the "references"  
24 (Chinnaiyan (NX 2058)<sup>7</sup> and Marsters (NX 2059)<sup>8</sup>), explain how percent identify

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<sup>7</sup> Chinnaiyan et al. (Chinnaiyan), "Signal Transduction by DR3, a Death Domain-Containing Receptor Related to TNFR-1 and CD95," Science, Vol. 274, pp. 990-992 (8 November 1996) (NX 2058).

1 scores based on different alignment methods and parameters relate to each  
2 other, what standard of error was typically found, whether iteration was  
3 necessary to obtain a statistically valid result, etc. 37 CFR § 41.158; Standing  
4 Order ¶¶ 24. Further, as illustrated by the discussion of Hb S above, even very  
5 small differences between protein variants with highly similar amino acid  
6 sequences can produce significant differences in function.

7       Therefore, in view of the state of the art at the time the '846 application  
8 was filed and the testimony of both Drs. Reed and Cheng, we find that the '846  
9 application does not describe an enabled embodiment (a functional DR5 having  
10 the deduced amino acid sequence shown in Figure 1) within the scope of Count  
11 1. The '846 application does describe a DR5 which may be preliminarily  
12 classified as a TNF death receptor protein based upon its deduced amino acid  
13 sequence. However, given the unpredictability of determining function from  
14 structure, a skilled artisan would have had to carry out further research to identify  
15 the function(s) of DR5 having the deduced amino acid sequence set forth in  
16 Figure 1.

17       Anticipation is a question of fact, not a conclusion of law, no matter how  
18 reasonable that conclusion may appear to be. Putative assignment to a protein  
19 (sub)family does not assess the actual biological function/utility of a nucleic acid  
20 sequence and its encoded protein product. Ni has failed to establish that the  
21 '846 application describes a functional death receptor protein within the scope of  
22 the count based solely on the disclosure of a deduced amino acid sequence.

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<sup>8</sup> Marsters et al. (Marsters), "Apo-3, a new member of the tumor necrosis factor receptor family, contains a death domain and activates apoptosis and NF- $\kappa$ B," Current Biology, Vol. 6, No. 12, pp. 1669-1676 (1996) (NX 2059).

1 Brenner v. Manson, 383 U.S. 519, 532, 148 USPQ 689, 694 (1966) ("the  
2 presumption that adjacent homologues have the same utility has been  
3 challenged in the steroid field because of 'greater known unpredictability of  
4 compounds in that field.'").

5 Ni also argues that the DR5 protein of the '846 application inherently binds  
6 TRAIL and that the '846 specification explicitly teaches that DR5 binds a TNF  
7 ligand selected from a limited list which includes TRAIL (Paper 30, p. 2, ¶ 3).  
8 However, before considering whether a limitation is an inherent characteristic of  
9 an embodiment within the scope of a count, that embodiment must itself be  
10 sufficiently described and enabled. Toro, 69 USPQ2d at 1590. Thus, this  
11 argument fails because Ni has not established that the '846 application describes  
12 an enabled embodiment within the scope of the count for the reasons above.  
13 Secondly, arguing that DR5 binds a TNF ligand from a limited list which includes  
14 TRAIL is also unpersuasive because the so-called "limited" list appears to cover  
15 all the known and unknown ligands of the TNF family, i.e., the list enumerates the  
16 eleven then known TNF ligands and then adds a catch-all "DR5 ligands,"  
17 seemingly in the event DR5 did not bind any of the then known TNF ligands.  
18 Neither the disclosure of the '846 application nor the testimony of Dr. Reed  
19 suggests that DR5 necessarily and always binds TRAIL or that DR5 binds a  
20 specific ligand from the "limited" subset of TNF ligands. Moreover, Ni's reliance  
21 on case law is misplaced.

22 Ni argues that  
23 even without express appreciation of a limitation  
24 recited in a count, disclosure in a priority application  
25 of an embodiment which is later shown to *inherently*

1 possess a characteristic satisfying that limitation is  
2 sufficient to establish constructive reduction to  
3 practice. *See e.g., Silvestri v. Grant*, 496 F.2d 593,  
4 599, 181 U.S.P.Q. 706, 710 (CCPA 1974) ("The  
5 invention is not the language of the count but the  
6 subject matter defined thereby."); *See also Hudziak v.*  
7 *Ring*, 2005 Pat. App. LEXIS 26 (Bd. Pat. App. Intf.,  
8 Sept. 2005) (confirming that a party's priority  
9 applications, which disclosed an antibody but did not  
10 state the antibody bound to a particular receptor  
11 protein (HER2) as recited in the count, were  
12 nonetheless constructive reductions to practice  
13 because subsequent evidence showed that the  
14 antibody bound HER2.) [Paper 30, p. 8, ¶ 1, original  
15 emphasis.]

16 Neither Silvestri nor Hudziak are on point. Silvestri has been discussed  
17 above (§III. Ni Substantive Motion 1). In Silvestri, the court held that the  
18 evidence established that Silvestri had prepared a new form of ampicillin,  
19 recognized and appreciated the existence of the new form of ampicillin, and that  
20 the new form of ampicillin had utility. *Id.*, 496 F.2d at 598-601, 181 USPQ at  
21 709-712. The court acknowledged that the ampicillin of the count required a  
22 molecular weight of about 349 and greater storage stability than the previously  
23 known form of ampicillin. However, the court thought these were inherent  
24 properties of the new form of ampicillin that Silvestri was said to have obtained,  
25 recognized and described. *Id.*, 496 F.2d at 599, 181 USPQ at 709. The court  
26 noted in Silvestri that the reduction to practice test does not require in haec verba  
27 appreciation of each of the limitations of the count:

28 This standard does not require that Silvestri establish  
29 that he recognized the invention in the same terms as  
30 those recited in the count. The invention is not the  
31 language of the count but the subject matter thereby  
32 defined. Silvestri must establish that he recognized  
33 and appreciated as a new form, a compound

1 corresponding to the compound defined by the count.  
2 Id., 496 F.2d at 599, 181 USPQ at 710

3 Here, the compound of the count is a functional protein which has at least  
4 90% identity to a defined amino acid sequence and binds TRAIL. Thus, it is  
5 necessary to consider whether the '846 application describes properties/uses of  
6 DR5. The '846 application only speculates that DR5 has desired properties, e.g.,  
7 inducing apoptosis upon activation. Ni is not in the same position as Silvestri  
8 whose application was said to have described obtaining an ampicillin compound,  
9 to have recognized it as a new form of ampicillin and to have described certain  
10 properties of the compound. Ni's '846 application describes a precursor to an  
11 encoded protein and speculates on the nature and properties of that protein.  
12 Therefore, Silvestri is not on point.

13 Similarly, in Hudziak v. Ring, 80 USPQ2d 1018, 1019 (Bd. Pat. App. & Int.  
14 2005), the count was directed to a monoclonal antibody that bound human  
15 epidermal growth factor receptor 2 (HER2). A panel of the Board decided that  
16 Chiron's (Ring's real party-in-interest) 1984 application disclosed an embodiment  
17 within the count, i.e., a murine monoclonal antibody designated 454C11. Id. The  
18 panel noted that the 1984 application (06/577,976) stated that hybridomas which  
19 produced 454C11 were deposited with the ATCC and that evidence submitted by  
20 Chiron established that 454C11 bound HER1. Id. at 1020-21.

21 57. The panel also noted in its decision (Paper 258, p. 129) that "Table 3 of  
22 the 1984 application reports the binding of antibodies to breast cancer  
23 cell lines and indicates that 454C11 binds to SKBR3 cells, which are now  
24 known to express HER2. (CX 1081, p. 3)."

1        Thus, in Hudziak, Chiron was said to have actually prepared an embodiment  
2        within the count, monoclonal antibody 454C11, and to have described it as a new  
3        protein and to have appreciated one of its properties/functions, i.e., that it bound  
4        to breast cancer cells. Ni's '846 application describes a precursor to an encoded  
5        protein and speculates on the nature and properties of that protein. Therefore,  
6        Hudziak is not on point.

7        Since Ni has failed to establish that the '846 application describes an  
8        enabled compound (functional DR5 protein) within the scope of the count, we do  
9        not reach the issue of what the inherent characteristics of that protein are. In  
10       both Silvestri and Hudziak, the application was said to specifically describe  
11       compounds that were recognized as novel and as having certain properties.  
12       These described and characterized compounds were later found to have other  
13       properties required by the count. Here, the '846 application does not describe  
14       and characterize a functional protein. Ni's application only speculates on the  
15       nature and properties of the protein encoded by the DNA of Figure 1 and that  
16       speculation is insufficient to show possession of an enabled embodiment within  
17       the count (which may later be found to have other properties required by the  
18       count).

19       Based on the foregoing, Ni is not entitled to benefit for the purpose of  
20       priority of the filing date of the '846 application as to Count 1.

21       In conclusion, Ni substantive motion 2 is **granted-in-part, denied-in-part**  
22       and **dismissed-in-part**.

23

1           **VI.       Rauch Substantive Motion 3**

2           Pursuant to 37 CFR § 41.121(a)(1)(iii) and the Order issued 29 November  
3   2005 (Paper 26), Rauch moves for judgment that Ni's '842 application claims 35,  
4   36, 38-45, 47-54, 56-61, 75, 83, 92, 99, 100, 102-109, 111-116, 127-133, 168-  
5   178 and 180-203 ("Ni's involved claims") are unpatentable under 35 U.S.C. §  
6   102(a) and/or (e) as clearly anticipated by one or more of U.S. Patent 6,642,358  
7   ("the '358 patent," RX 1042), U.S. Patent 6,072,047 ("the '047 patent," RX 1048),  
8   U.S. Patent 6,569,642 ("the '642 patent," RX 1046) and WO 98/35986 ("WO  
9   '986," RX 1032) (collectively, "the Rauch references") (Paper 36, p. 25). Ni  
10   opposes (Paper 49); Rauch replies (Paper 66).

11           58. According to the '358 patent, it issued 4 November 2003 based on  
12           application 09/578,392, filed 25 May 2000, which is a divisional of  
13           application 08/883,036, filed 26 June 1997, which is a continuation-in-  
14           part of application 08/869,852, filed 4 June 1997, which is a continuation-  
15           in-part of application 08/829,536, filed 28 March 1997, which is a  
16           continuation-in-part of application 08/815,255, filed 12 March 1997, which  
17           is a continuation-in-part of application 08/799,861, filed 13 February 1997  
18           (RX 1042, title page).

19           59. According to the '047 patent, it issued 6 June 2000 based on application  
20           08/883,036, filed 26 June 1997, which is a continuation-in-part of  
21           application 08/869,852, filed 4 June 1997, which is a continuation-in-part  
22           of application 08/829,536, filed 28 March 1997, which is a continuation-  
23           in-part of application 08/815,255, filed 12 March 1997, which is a

1 continuation-in-part of application 08/799,861, filed 13 February 1997  
2 (RX 1048, title page).

3 60. According to the '642 patent, it issued 27 May 2003 based on application  
4 09/536,201, filed 27 March 2000, which is a continuation-in-part of  
5 application 08/883,036, filed 26 June 1997, which is a continuation-in-  
6 part of application 08/869,852, filed 4 June 1997, which is a continuation-  
7 in-part of application 08/829,536, filed 28 March 1997, which is a  
8 continuation-in-part of application 08/815,255, filed 12 March 1997, which  
9 is a continuation-in-part of application 08/799,861, filed 13 February 1997  
10 (RX 1046, title page).

11 61. WO '968 published 20 August 1998, based on international application  
12 PCT/US98/02239, filed 11 February 1998 (RX 1032, title page).

13 According to the relevant paragraphs of 35 U.S.C. § 102:

14 [a] person shall be entitled to a patent unless--

15 (a) the invention was known or used by others  
16 in this country, or patented or described in a printed  
17 publication in this or a foreign country before the  
18 invention thereof by the applicant for patent, or

19 \* \* \* \* \*

20 (e) the invention was described in (1) an  
21 application for a patent, published under section  
22 122(b), by another filed in the United States before  
23 the invention by the applicant for patent or (2) a  
24 patent granted on an application for patent by another  
25 filed in the United States before the invention by the  
26 applicant for patent, except that an international  
27 application filed under the treaty defined in section  
28 351(a) shall have the effects for the purposes of this  
29 subsection of an application filed in the United States  
30 only if the international application designated the



1 United States and was published under Article 21(2)  
2 of such treaty in the English language, or

3 \* \* \* \* \*

4 References based on international applications that were filed prior to 29  
5 November 2000 are subject to the former version of 35 U.S.C. § 102(e),<sup>9</sup> i.e.,

6 [a] person shall be entitled to a patent unless --

7 (e) the invention was described in a patent  
8 granted on an application for patent by another filed in  
9 the United States before the invention thereof by the  
10 applicant for patent, or on an international application  
11 by another who has fulfilled the requirements of  
12 paragraphs (1), (2), and (4) of section 371(c) of this  
13 title before the invention thereof by the applicant for  
14 patent.

15 A prima facie case is made out under § 102(a) if, within a year of the filing  
16 date, the invention, or an obvious variant thereof, is described in a "printed  
17 publication" whose authorship differs from the inventive entity unless it is stated  
18 within the publication itself that the publication is describing the applicant's work.  
19 In re Katz, 687 F.2d 450, 215 USPQ 14 (CCPA 1982).

20 62. None of the Rauch references issued or published prior to the 17 March  
21 1998 filing date of the Ni claims at issue.<sup>10</sup>

22 63. None of the Rauch references qualify as prior art under § 102(a) vis-à-vis  
23 the Ni claims at issue.

24 Therefore, to the extent Rauch substantive motion 3 seeks a judgment that  
25 any of the Ni claims at issue are unpatentable under § 102(a) as anticipated by

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<sup>9</sup> Pursuant to § 13205 of Pub. L. 107-273.

<sup>10</sup> Rauch has not argued prior knowledge or use of the subject matter of any of the Ni claims at issue.

1 any of the Rauch references, the motion is **denied**. We now consider whether  
2 any of the Rauch references qualify as prior art under § 102(e).

3 WO '986 is based on an international application filed prior to 29 November  
4 2000 (FF 61). Therefore, it must satisfy the requirements of the then applicable  
5 former § 102(e) in order to qualify as prior art. Rauch has neither argued nor  
6 shown that WO '986 satisfies the requirements of the applicable § 102(e) (see  
7 Paper 36, p. 22, ¶ 2). Thus, Rauch has not established that WO '986 qualifies as  
8 prior art under the applicable § 102(e) vis-à-vis the Ni claims at issue.  
9 Consequently, to the extent Rauch substantive motion 3 seeks a judgment that  
10 any of the Ni claims at issue are unpatentable under § 102(e) as anticipated by  
11 WO '986, the motion is **denied**.

12 As indicated above (FFs 58-60), the '358, '047 and '642 patents are related.  
13 The '047 patent issued based on application 08/833,036 and the '358 and '642  
14 patents issued based on an application identified as a divisional or a  
15 continuation-in-part, respectively, of application 08/833,036, filed on 26 June  
16 1997. The filing date of the 08/833,036 application is prior to the 17 March 1998  
17 filing date of Ni's involved claims and prima facie qualifies as prior art under  
18 § 102(e) against the Ni claims at issue. It is not necessary to consider whether  
19 the Ni claims at issue are anticipated by the '358 and '642 patents, if the Ni claims  
20 at issue are anticipated by the '047 patent.

21 Claim chart appendix I attached to Rauch substantive motion 3 (Paper 36,  
22 beginning at p. 243) correlates the disclosure of the '047 patent to each of the  
23 limitations of each of the Ni claims at issue. Therefore, Rauch substantive

1 motion 3, when considered in light of the evidence relied upon in support of the  
2 motion, establishes a sufficient basis for holding the Ni claims at issue prima  
3 facie unpatentable under § 102(e) as anticipated by the '047 patent.

4 As noted by Rauch in its reply (Paper 66, p. 6, ¶ 1), Ni does not contest that  
5 the '047 patent describes the subject matter of its claims at issue. Rather, Ni  
6 argues that the '047 patent does not qualify as prior art because Ni's '583  
7 application claims are said to be entitled to benefit of the 17 March 1997 filing  
8 date of Ni's '846 application (Paper 49, p. 2, ¶ 2; ¶ bridging pp. 24-25; Appendix  
9 E).<sup>11</sup> Rauch maintains that Ni cannot obtain benefit of the filing date of its '846  
10 application due to a lack of utility (Paper 36, p. 22, ¶ 3 through p. 24, ¶ 1).

11 As stated in In re Fisher, 421 F.3d 1365, 1378, 76 USPQ2d 1225, 1235  
12 (Fed. Cir. 2005),

13 [i]t is well established that the enablement  
14 requirement of § 112 incorporates the utility  
15 requirement of § 101. The how to use prong of  
16 section 112 incorporates as a matter of law the  
17 requirement of 35 U.S.C. § 101 that the specification  
18 disclose as a matter of fact a practical utility for the  
19 invention. If the application fails as a matter of fact to  
20 satisfy 35 U.S.C. § 101, then the application also fails  
21 as a matter of law to enable one of ordinary skill in the  
22 art to use the invention under 35 U.S.C. § 112.

23 The dispositive question here is whether the Ni claims at issue are entitled to  
24 benefit of the 17 March 1997 filing date of Ni's '846 provisional application,  
25 thereby, antedating the 26 June 1997 filing date of the '047 patent. Benefit for  
26 purposes of antedating prior art, in this case, benefit under 35 U.S.C. § 119(e), is

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<sup>11</sup> We need not consider whether Ni's '842 application claims are entitled to § 119(e) benefit of the 17 March 1998 filing date of Ni's '583 application or the 29 July 1997 filing date of Ni's '021 application because both of these two filing dates are after the 26 June 1997 filing date of the 08/833,036 application which issued as Rauch's '047 patent.

1 different from benefit for the purpose of priority. To obtain benefit of the filing  
2 date of a provisional application under § 119(e), the provisional application must,  
3 in relevant part, satisfy the description and enablement requirements of § 112,  
4 first paragraph, for the full scope of the claimed subject matter for which benefit is  
5 being sought. Ni and Rauch disagree as to whether the disclosure of Ni's '846  
6 provisional application satisfies the description and enablement requirements of  
7 § 112, first paragraph, as to the full scope of the subject matter of the Ni claims at  
8 issue.

9 Ni cites to specific disclosures in its '846 application said to describe every  
10 element of its claims at issue (Appendix E attached to Paper 49). Ni argues that  
11 the '846 application discloses that DR5 polypeptides are useful (a) to make anti-  
12 DR5 antibodies for treating or diagnosing diseases associated with apoptosis or  
13 (b) as antagonists of DR5 signaling (Paper 49, p. 7, ¶¶ 1-2).

14 64. Dr. Reed, testifying for Ni, stated that the technology necessary to  
15 achieve these functions was within routine skill in the art, e.g., a skilled  
16 artisan would know how to express and purify a protein (e.g., DR5) from  
17 cDNA (e.g., DNA of Figure 1 in the '846 application), how to produce  
18 antibodies that bind to a desired protein (e.g., DR5), etc. (NX 2103, ¶¶  
19 35-46).

20 65. Dr. Reed further testified that the uses for DR5 described in the '846  
21 application would have been believable to one of ordinary skill in the art  
22 because the asserted uses had previously been shown to be recognized

1           uses of TNF death receptors TNFR1, Fas and/or DR3 (NX 2103, ¶¶ 33-  
2           34 and 47-52).

3           Essentially, Dr. Reed's testimony as to the utility/enablement of DR5 is  
4           based on the assumption that the DR5 described in the '846 application is a  
5           functional TNF death receptor protein and, therefore, what was known about the  
6           use of other TNF death receptors was directly applicable to DR5 (see e.g., NX  
7           2103, ¶¶ 49 and 50 ("[b]ased on precedent from prior work in the field of TNF-  
8           family receptors" and "[b]ased on precedent from the literature where agonistic  
9           and antagonistic antibodies to other TNF-family receptors had been produced  
10          and characterized," respectively)). According to Ni, Dr. Reed "has testified  
11          unequivocally that 'you can reasonably make a prediction based on homology  
12          alone' and by analyzing 'the particular subfamily of proteins to which DR5  
13          belongs, *i.e.*, death receptors", "the most reasonable conclusion to draw from Ni's  
14          March 17, 1997 application is that DR5 is expected, by persons of ordinary skill  
15          in the art to be a novel death receptor [and that] a person of ordinary skill in the  
16          art would have predicted that activation of DR5 would induce apoptosis" (Paper  
17          49, p. 10, ¶ 1, citations omitted). The disclosure cited by Ni in its Appendix I is no  
18          more specific than Dr. Reed's testimony. For example, in the third paragraph of  
19          the third column on page 1 of Appendix I, Ni points to p. 6, lines 25-34 of the '846  
20          application as disclosing that "[t]he homology DR5 shows to other death domain  
21          containing receptors strongly indicates that DR5 is also a death domain  
22          containing receptor with the ability to induce apoptosis." Thus, according to Ni,  
23          Dr. Reed properly focused on the subset of known death receptors and the

1 "single" function that unites them, i.e., their ability to induce apoptosis (Paper 49,  
2 pp. 9-10).

3 Rauch, on the other hand, argues that sequence homology alone is  
4 insufficient to establish that the DR5 polypeptide disclosed in the '846 application  
5 is in fact a TNF family death domain receptor. According to Rauch, unless the  
6 disclosure of the '846 application shows DR5 to be an actual TNF family member  
7 receptor, e.g., by identification of a known TNF ligand as its cognate ligand or by  
8 specific experimental data showing that DR5 induces a TNFR-mediate biological  
9 activity, e.g., apoptosis, inflammatory response, etc., the '846 application fails to  
10 disclose a specific, substantial and credible utility for the DR5 and, therefore, for  
11 the Ni claims at issue (Paper 36, ¶¶ bridging pp. 23-24).

12 66. Dr. Cheng testified for Rauch that Ni's '846 application discloses  
13 the DNA and amino acid sequence of the 411 amino  
14 acid isoform of TR-2, which they refer to as DR5.  
15 DR5 was identified based on sequence homology to  
16 other death domain-containing members of the TNFR  
17 family, including TNFR-1, DR3, and Fas ('846  
18 Provisional, page 5, lines 21-24). The applicants  
19 assert that agonists to DR5 can be used to increase  
20 apoptosis, while antagonists to DR5 can be used to  
21 inhibit apoptosis. This assertion is based entirely on  
22 sequence homology between DR5 and death domain-  
23 containing receptors TNFR-1, DR3, and Fas.  
24 However, the '846 Provisional does not identify a  
25 ligand for DR5, and contains no experimental data  
26 regarding DR5 function.

27 Sequence homology to other death domain-  
28 containing TNF receptors may be sufficient to  
29 convince one of ordinary skill in the art that a novel  
30 protein is a TNFR family member. However,  
31 sequence homology alone is not sufficient to support  
32 an assertion that a novel TNFR family member  
33 protein will induce specific biological activities such as  
34 apoptosis. Without additional data regarding the

1 activity of a TNFR family member, such as, for  
2 example, the identity of the ligand with a known  
3 function (such as TRAIL) to which it binds, one of  
4 ordinary skill in the art cannot reasonably predict the  
5 function of the TNFR family member. This is because  
6 TNFR family members are involved in complex signal  
7 transduction pathways which can affect a wide  
8 spectrum of biological activities including apoptosis,  
9 inflammatory response, cell proliferation, cell survival  
10 and other activities. The binding of certain TNFR  
11 family members by their corresponding ligands can  
12 lead to activation of multiple signal transduction  
13 pathways. As stated above, the '846 Provisional  
14 contains no data regarding the ligand for DR5, nor  
15 does it disclose experimental data of its function.  
16 Without knowing more information about the activity  
17 of DR5, such as for example its specificity for a ligand  
18 with a known function, one of ordinary skill in the art  
19 could not reasonably predict the function of the TNFR  
20 family member protein. [RX 1039, ¶¶ 16-17.]

21 For essentially the reasons set forth in our analysis in "§VI. Ni Substantive  
22 Motion 2" above, we credit the testimony of Dr. Cheng over that of Dr. Reed. In  
23 short, one of ordinary skill in the art might classify DR5 as disclosed in Ni's '846  
24 application as a possible TNF death receptor protein based on the similarity  
25 between its deduced amino acid sequence and the known amino acid sequences  
26 of TNF death receptor proteins TNFR1, Fas and DR3. However, given the  
27 unpredictability of determining function from structure (the "Holy Grail" of  
28 molecular biology), a skilled artisan would have had to carry further research to  
29 identify the function(s) of a DR5 polypeptide having the deduced amino acid  
30 sequence set forth in Figure 1 of the '846 application. Thus, the disclosure of the  
31 '846 application fails to satisfy the "how-to-use" requirement of § 112, first  
32 paragraph, as to the subject matter of the Ni claims at issue. The Ni claims at  
33 issue are, therefore, not entitled to § 119(e) benefit of the filing date of Ni's '846

1 application and Rauch's '047 patent still qualifies as prior art under § 102(e).  
2 Therefore, Ni claims 35, 36, 38-45, 47-54, 56-61, 75, 83, 92, 99, 100, 102-109,  
3 111-116, 127-133, 168-178 and 180-203 of Ni's '842 application (the Ni claims at  
4 issue) are unpatentable under 35 U.S.C. § 102(e) as anticipated by U.S. Patent  
5 6,072,047. It is not necessary to our decision to consider whether the Ni claims  
6 at issue are also anticipated by either the '358 or '642 patent.

7 In its opposition, Ni also argues that Rauch substantive motion 3 should  
8 be denied on procedural grounds because it does not seek judgment that all of  
9 Ni's involved claims are unpatentable and, therefore, is not a proper threshold  
10 motion (Paper 49, p. 13, ¶ 2 - p. 14, ¶ 1). Rauch substantive motion 3 is an  
11 ordinary attack on patentability. Ni has not provided any basis requiring a motion  
12 for unpatentability to attack all of a party's involved claims and we know of none.  
13 Therefore, this argument is without merit.

14 Based on the foregoing, Rauch substantive motion 3 is **granted only to**  
15 **the extent** that Ni claims 35, 36, 38-45, 47-54, 56-61, 75, 83, 92, 99, 100, 102-  
16 109, 111-116, 127-133, 168-178 and 180-203 are unpatentable under 35 U.S.C.  
17 § 102(e) as anticipated by U.S. Patent 6,072,047.

18 **VII. Rauch Substantive Motion 2**

19 Pursuant to 37 CFR § 41.121(a)(1)(i), Rauch moves to redefine the scope  
20 of the interference by designating Ni claims 46, 55, 63, 64, 110 and 118 of the  
21 '842 application as corresponding to Count 1 (Paper 35). Ni opposes (Paper 48);  
22 Rauch replies (Paper 65).

23 67. Ni '842 application claim 46, written in independent form, reads



- 1                   An isolated polypeptide comprising an amino acid  
2                   sequence at least 95% identical to amino acids -50 to  
3                   360 of SEQ ID NO:2, wherein said polypeptide  
4                   induces apoptosis.
- 5           68. Ni '842 application claim 55, written in independent form, reads:  
6                   An isolated polypeptide comprising an amino acid  
7                   sequence at least 95% identical to amino acids -51 to  
8                   360 of SEQ ID NO:2, wherein said polypeptide  
9                   induces apoptosis.
- 10          69. Ni '842 application claim 63, written in independent form, reads:  
11                   An isolated polypeptide comprising amino acids -50 to  
12                   360 of SEQ ID NO:2.
- 13          70. Ni '842 application claim 64, written in independent form, reads:  
14                   An isolated polypeptide comprising amino acids -51 to  
15                   360 of SEQ ID NO:2.
- 16          71. Ni '842 application claim 110, written in independent form, reads:  
17                   An isolated polypeptide comprising an amino acid  
18                   sequence at least 95% identical to the full length  
19                   amino acid sequence encoded by the cDNA clone in  
20                   ATCC Deposit No. 97920, wherein said polypeptide  
21                   induces apoptosis.
- 22          72. Ni '842 application claim 118, written in independent form, reads:  
23                   An isolated polypeptide comprising the full length  
24                   amino acid sequence encoded by the cDNA clone in  
25                   ATCC Deposit No. 97920.
- 26          73. SEQ ID NO:2 of Rauch's involved '358 patent contains 440 amino acid  
27                   residues.
- 28          74. Amino acid residues 1 to 440 of Rauch '358 patent are identical to amino  
29                   acid residues -51 to 360 of SEQ ID NO:2 of Ni's 842 application except  
30                   for the inclusion of additional amino acid residues 185 to 213 in SEQ ID  
31                   NO: 2 of Rauch's '358 patent (RX 1040, pp. 24-25 and RX 1042, ccs. 33-  
32                   35).

1       75. According to Ni's '842 specification, the polypeptide encoded by the  
2       cDNA clone in ATCC Deposit No. 97920 has the amino acid sequence  
3       recited in SEQ ID NO:2 (RX 1040, p. 4, ll. 18-21; p. 9, ll. 5-8 and 13-17).

4       76. Further according to Ni's '842 specification, the full length DR5 lacks the  
5       methionine encoded by nucleotides 130-132 of SEQ ID NO: 1 (RX 1040,  
6       p. 11, ll. 28-32) and "may or may not include the leader sequence" (id., p.  
7       37, ll. 15-16).

8       Rauch argues that

9       a DNA sequence encoding a polypeptide "at least  
10       90% identical" to Rauch SEQ ID NO:2 would include  
11       (1) a DNA sequence encoding a polypeptide having  
12       the same sequence as residues 1 to 440 of Rauch  
13       SEQ ID NO:2; (2) a DNA sequence encoding a  
14       polypeptide having the same sequence as residues 1  
15       to 440 of Rauch SEQ ID NO:2 but for the substitution  
16       of 1 to 44 of the 440 residues; (3) a DNA sequence  
17       encoding a polypeptide having the same sequence as  
18       residues 1 to 440 of Rauch SEQ ID NO:2 but for the  
19       deletion of 1 to 44 residues; and (4) a DNA sequence  
20       encoding a polypeptide having the same sequence as  
21       residues 1 to 440 of Ni [sic] SEQ ID NO:2 but for the  
22       addition of 1 to 44 additional residues to the 440  
23       residues. [Paper 35, p. 5, ll. 1-10.]

24       In essence, Rauch's position is that "as long as a single species of a claim falls  
25       within the count, then that claim corresponds to the count" (id., p. 5, ll. 14-15).

26       "A claim corresponds to a count if the subject matter of the count, treated  
27       as prior art to the claim, would have anticipated or rendered obvious the subject  
28       matter of the claim." 37 CFR § 41.207(b)(2). A prior art species within a claimed  
29       genus reads on the generic claim and anticipates. In re Gostelli, 872 F.2d 1008,  
30       1010, 10 USPQ2d 1614, 1616 (Fed. Cir. 1989). However, a species claim is not  
31       necessarily obvious in light of a prior art disclosure of a genus. In re Baird, 16

1 F.3d 380, 382, 29 USPQ2d 1550, 1552 (Fed. Cir. 1994). In other words, the  
2 "earlier disclosure of a genus does not necessarily prevent the patenting of a  
3 species member of that genus." Eli Lilly & Co. v. Bd. of Regents of the Univ. of  
4 Washington, 334 F.3d 1264, 1270, 67 USPQ2d 1161, 1165 (Fed. Cir. 2003)  
5 (citations omitted).

6 Here, the subject matter of Count 1 is directed to a genus of functional  
7 proteins, i.e., purified TRAIL-R polypeptides having an amino acid sequence that  
8 is at least 90% identical to SEQ ID NO:2 of Rauch's involved '358 patent,  
9 wherein the polypeptides bind TRAIL (FF 11). Assuming without deciding that  
10 the isolated polypeptides of Ni claims 46, 55, 63, 64, 110 and 118 bind TRAIL,  
11 none of these claims recite an isolated polypeptide having an amino acid  
12 sequence identical to SEQ ID NO:2 of Rauch's '358 patent. Ni claims 46, 55, 63,  
13 64, 110 and 118 are directed to subgenera/species within the genus of Count 1.  
14 The genus of Count 1 does not anticipate the specific subgenera/species of Ni  
15 claims 46, 55, 63, 64, 110 and 118. For example, Ni claim 46 recites a subgenus  
16 (an isolated polypeptide comprising an amino acid sequence at least 95%  
17 identical to) within a subgenus (amino acids -50 to 360 of SEQ ID NO:2 of Ni's  
18 '842 application, wherein said polypeptide induces apoptosis) (FF 67). Simply  
19 showing that a subgenus/species claim falls within the subject matter of a generic  
20 count does not suffice to establish that the claim is anticipated or rendered  
21 obvious by the subject matter of the count. Rauch has not established why any  
22 of Ni claims 46, 55, 63, 64, 110 and 118 would be unpatentable over the subject  
23 matter of Count 1, i.e., why the subject matter of each of these claims is an

1 obvious subgenera/species within the generic subject matter of the count.

2 Therefore, Rauch has failed to meet its burden.

3 Based on the foregoing, Rauch substantive motion 2 is **denied**.

4 **VIII. Rauch Substantive Motion 1**

5 Pursuant to 37 CFR § 41.121(a)(1)(ii), Rauch moves to be accorded  
6 benefit for the purpose of priority of the (i) 13 February 1997 filing date of  
7 application 08/799,861 ("the '861 application," RX 1014), (ii) 12 March 1997 filing  
8 date of application 08/815,255 ("the '255 application," RX 1015), (iii) 28 March  
9 1997 filing date of application 08/829,536 ("the '536 application," RX 1016), and  
10 (iv) 4 June 1997 filing date of application 08/869,852 ("the '852 application," RX  
11 1017) (Paper 34). Ni opposes (Paper 47); Rauch replies (Paper 64).

12 The '392 application from which Rauch's involved '358 patent issued has  
13 already been accorded benefit of the 26 June 1997 filing date of Rauch's earlier  
14 filed '036 application (FF 7).

15 77. The '036 application is a continuation-in-part of the '852 application,  
16 which is a continuation-in-part of the '536 application, which is a  
17 continuation-in-part of the '255 application, which is a continuation-in-part  
18 of the '861 application (RX 1042, title page).

19 Benefit for the purpose of priority focuses on the subject matter of a count  
20 and only requires a constructive reduction to practice of a single embodiment  
21 within the scope of the count. Here, the subject matter of the count is directed to  
22 a purified TRAIL-R polypeptide having an amino acid sequence that is at least

1 90% identical to SEQ ID NO:2 of Rauch's involved '358 patent, wherein the  
2 polypeptide binds TRAIL (FF 11).

3 Rauch contends that the two earliest ('861 and '255) applications disclose a  
4 method of obtaining and purifying TRAIL-R protein, its ability to bind TRAIL, its  
5 molecular weight and partial amino acid sequences thereof sufficient to convince  
6 one of ordinary skill in the art that Rauch had possession of an isolated, purified  
7 TRAIL-R protein which inherently has an amino acid sequence at least 90%  
8 identical to SEQ ID NO:2 of the '358 patent (Paper 34, pp. 5-8). Rauch further  
9 contends that the later two ('536 and '852) applications additionally disclose the  
10 full-length amino acid sequence of TRAIL-R which is identical to the amino acid  
11 sequence set forth in SEQ ID NO:2 of the '358 patent (Paper 34, pp. 8-10).

12 Ni argues that none of the four applications disclose any utility for TRAIL-R  
13 protein and, therefore, fail the how-to-use prong of the enablement requirement  
14 of 35 U.S.C. § 112, first paragraph (Paper 47, p. 2). As to the two earliest ('861  
15 and '255) applications, Ni further argues that (a) the disclosed purification method  
16 results in a mixture of TRAIL-binding proteins, (b) the disclosed partial amino  
17 acid sequence contains amino acids not present in SEQ ID NO:2 of the '358  
18 patent, (c) the disclosed molecular weight is insufficient to differentiate TRAIL-R  
19 protein from other TRAIL-binding proteins, and (d) the amino acid sequence of  
20 the "purified" protein is less than 90% identical to SEQ ID NO:2 of the '358 patent  
21 (Paper 47, pp. 8-22).

22 78. It is undisputed that the TRAIL-R protein having the amino acid sequence  
23 set forth in SEQ ID NO:2 of the '358 patent is the 440 amino acid

1 isoform<sup>12</sup> of a TNF receptor protein alternatively referred to in the art as  
2 TR-2, DR5, Apo-2, TRICK2 and KILLER (see Paper 47, p. B-1 where Ni  
3 admits Rauch SMFs 1 and 6 as set forth in Paper 34, p. 12).

4 79. According to the '358 patent, the TRAIL-R protein of SEQ ID NO:2 is a  
5 full-length protein which includes an N-terminal signal peptide<sup>13</sup> (RX  
6 1042, c. 2, ll. 54-56).

7 80. Further according to the '358 patent, the signal peptide of the 440 amino  
8 acid full-length TRAIL-R protein is predicted to correspond to amino acids  
9 1 to 51 or 1 to 56 of SEQ ID NO:2 (RX 1041, c. 2, ll. 58-62; c. 3., ll. 1-12).

10 **A. The '852 (RX 1017) and '536 (RX 1016) applications**

11 81. According to the '852 specification, TRAIL or "TNF-related apoptosis-  
12 inducing ligand" is a member of the tumor necrosis factor (TNF) family of  
13 ligands and TRAIL-R binds TRAIL (RX 1017, p. 1, ll. 16-18 and 26-28; p.  
14 2, ll. 9-10).

15 82. According to the '536 specification, TRAIL or "TNF-related apoptosis-  
16 inducing ligand" is a member of the tumor necrosis factor (TNF) family of  
17 ligands and TRAIL-R binds TRAIL (RX 1016, p. 1, ll. 15-17 and 25-27; p.  
18 2, ll. 9-10).

19 83. Further according to the '852 specification, "[c]ertain uses of TRAIL-R  
20 flow from this ability to bind TRAIL, . . . TRAIL-R finds use in inhibiting

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<sup>12</sup> An isoform is a variant of the same protein between various tissues, development stages, etc. with some small differences, usually a splice variant or the product of some posttranslational modification.

<sup>13</sup> A signal peptide (or leader sequence) is a continuous sequence of amino acids, normally at the N-terminus of a protein, that targets the full-length protein to its eventual location in a cell and is then cleaved off (see generally, MCB, p. 652) (copy enclosed).

- 1 biological activities of TRAIL, or in purifying TRAIL by affinity  
2 chromatography, for example" (RX 1017, p. 2, ll. 10-12; these and  
3 additional uses are set forth at p. 20, l. 15 - p. 25, l. 11).
- 4 84. Further according to the '536 specification, "[c]ertain uses of TRAIL-R  
5 flow from this ability to bind TRAIL, . . . TRAIL-R finds use in inhibiting  
6 biological activities of TRAIL, or in purifying TRAIL by affinity  
7 chromatography, for example" (RX 1016, p. 2, ll. 10-12; these and  
8 additional uses are set forth at p. 13, l. 34 - p. 18, l. 26).
- 9 85. Example 6 in the '852 specification is said to demonstrate the ability of  
10 full length human TRAIL-R to bind TRAIL (RX 1017, p. 35, l. 4 - p. 36, l.  
11 13).
- 12 86. The '536 specification explicitly states that TRAIL-R binds TRAIL (RX  
13 1016, p. 1, ll. 25-27; p. 13, l. 36; p. 22, l. 25 - p. 23, l. 22).
- 14 87. SEQ ID NO:1 of the '852 application is said to show a human foreskin  
15 fibroblast derived TRAIL-R cDNA encoding a protein having the amino  
16 acid sequence set forth in SEQ ID NO:2 of the '852 application (RX 1017,  
17 p. 33, ll. 17-21; pp. 39-43).
- 18 88. Figure 2 of the '536 application is said to show a human foreskin  
19 fibroblast derived TRAIL-R cDNA encoding a protein having the amino  
20 acid sequence set forth in Figure 3 of the '536 application (RX 1016, p.  
21 24, ll. 29-33).
- 22 89. It is undisputed that the full length TRAIL-R amino acid sequence set  
23 forth in SEQ ID NO:2 of the '852 application is identical to the full length

1 TRAIL-R amino acid sequence set forth in SEQ ID NO:2 of the '358  
2 patent (compare RX 1017, pp. 42-43, and RX 1042, cc. 33-35; see Paper  
3 47, p. B-3 where Ni admits Rauch SMF 27 as set forth in Paper 34, p.  
4 17).

5 90. It is undisputed that the full length TRAIL-R amino acid sequence set  
6 forth in Figure 3 of the '536 application is identical to the full length  
7 TRAIL-R amino acid sequence set forth in SEQ ID NO:2 of the '358  
8 patent (compare RX 1016, Figure 3, and RX 1042, cc. 33-35; see Paper  
9 47, p. B-3 where Ni admits Rauch SMF 24 as set forth in Paper 34, p.  
10 17).

11 91. Thus, the '852 and '536 applications each describe an embodiment within  
12 the scope of Count 1, i.e., a a purified TRAIL-R polypeptide having an  
13 amino acid sequence that is at least 90% identical to SEQ ID NO:2 of  
14 Rauch's involved '358 patent (FFs 87-90), wherein the polypeptide binds  
15 TRAIL (FFs 81-86).

16 Relying on Rasmusson v. SmithKline Beecham Corp., 413 F.3d 1318, 75  
17 USPQ2d 1297 (Fed. Cir. 2005), Ni argues that neither the '852 nor the '536  
18 application discloses any utility for TRAIL-R protein and, therefore, fail the how-  
19 to-use prong of the enablement requirement of 35 U.S.C. § 112, first paragraph  
20 (Paper 47, p. 2 and p. 7, ¶ 2). Specifically, Ni argues that "[n]owhere in Rauch  
21 Substantive Motion 1 does Party Rauch even imply that its earlier applications  
22 discloses [sic] a utility for a polypeptide of the count" (Paper 47, p. 7, ¶ 4).



1           In essence, the only opposition raised by Ni is whether the '852 and '536  
2   applications disclose an adequate utility/enablement for a polypeptide within the  
3   scope of the count. First, Count 1 explicitly describes a utility for a polypeptide  
4   within its scope, i.e., the polypeptide binds TRAIL. Second, Rauch asserted this  
5   utility/enablement (Paper 34, p. 8, ¶ 3 - p. 10, ¶ 1) and pointed to express  
6   descriptive support of an embodiment within the scope of Count 1 in the '852 and  
7   '536 applications in Appendices F and E, respectively, of its motion. Third, the  
8   '852 and '536 specifications explicitly state that TRAIL-R binds TRAIL (FFs 81-  
9   86). Fourth, our finding that the '852 and '536 applications describe and enable  
10   an embodiment within the scope of Count 1 is not inconsistent with the holding in  
11   Rasmusson.

12           In Rasmusson both parties had interfering claims directed to methods of  
13   treating prostate cancer comprising administering finasteride, a selective 5- $\alpha$ -  
14   reductase inhibitor. An interference was declared by the Board of Patent  
15   Appeals and Interferences (the Board). Rasmusson was involved in the  
16   interference on the basis of an application which claimed priority to eight earlier  
17   filed applications. SmithKline Beecham Corp. was involved in the interference on  
18   the basis of two patents and corresponding reissue applications. On appeal from  
19   the decision of the Board, the Federal Circuit affirmed the Board's holding that  
20   Rasmusson was not entitled to benefit for the purpose of priority of the filing  
21   dates of the eight earlier filed applications. Citing In re Brana, 51 F.3d 1560, 34  
22   USPQ2d 1436 (Fed. Cir. 1995), the court said "a specification disclosure which  
23   contains a teaching of the manner and process of making and using the invention

1 . . . must be taken as in compliance with the enabling requirement of the first  
2 paragraph of § 112 unless there is a reason to doubt the objective truth of the  
3 statements contained therein which must be relied on for enabling support"  
4 (Rasmusson, 413 F.3d at 1323, 75 USPQ2d at 1300, emphasis added). The  
5 court affirmed the Board's finding that one of ordinary skill in the art would not  
6 have believed that finasteride was effective in treating prostate cancer in light of  
7 the state of the art at the relevant time and because Rasmusson had failed to  
8 provide experimental proof demonstrating the effectiveness of the invention (id.,  
9 413 F.3d at 1324-25, 75 USPQ2d at 1301).

10 Here, the '852 and '536 specifications explicitly state that TRAIL-R binds  
11 TRAIL (FFs 81-82). The '852 and '536 specifications further describe certain  
12 uses of TRAIL-R based on its ability to bind TRAIL, e.g., using TRAIL-R to purify  
13 TRAIL by affinity chromatography (FFs 83-84). Ni has not pointed to evidence of  
14 record which raises doubts as to the objective truth of these statements in either  
15 the '852 or '536 specifications, as was the case in Rasmusson. For example, Ni  
16 does not argue or provide evidence that a receptor protein that binds a ligand  
17 could not be used to purify the ligand by affinity chromatography at the time the  
18 '852 or '536 application was filed. Alternatively, Ni does not provide any  
19 evidence that the TRAIL-R protein set forth in SEQ ID NO:2 and Figure 3 of the  
20 '852 and '536 applications, respectively, does not bind TRAIL. Moreover, Ni  
21 does not argue that the '852 and '536 applications fail to disclose any utility for  
22 the TRAIL-R polypeptide set forth their respective SEQ ID NO:2 and Figure 3  
23 (FFs 87-88). In short, Rauch has described how to use a purified TRAIL-R

1 polypeptide within the scope of the count, i.e., TRAIL-R binds TRAIL (Paper 34,  
2 p. 8, ¶ 3 - p. 10, ¶ 1), and Ni has not provided any basis to doubt the objective  
3 truth of express statements in the '852 and '536 specifications that the TRAIL-R  
4 of their respective SEQ ID NO:2 and Figure 3 is useful to bind TRAIL.

5 Based on the foregoing, Rauch substantive motion 1 is **granted** as to the  
6 '852 and '536 applications.

7 **B. The '255 (RX 1015) and '861 (RX 1014) applications**

8 92. According to the '255 specification, TRAIL-R is a protein which binds  
9 TRAIL and, thus, finds uses in affinity chromatography purification of  
10 TRAIL and in inhibiting biological activities of TRAIL (RX 1015, p. 1, ¶ 5).

11 93. The '255 specification states that Example 1 discloses the isolation and  
12 purification of human TRAIL-R protein with a molecular weight of about  
13 52 kD from the cell membranes of Jurkat cells (RX 1015, p. 16, ¶ 3).

14 94. Specifically, "Jurkat cells are disrupted, and the subsequent purification  
15 process includes affinity chromatography (employing a chromatography  
16 matrix containing TRAIL), and reversed phase HPLC" (RX 1015, p. 4, ¶  
17 5).

18 95. Further according to the '255 specification, Example 2 discloses the  
19 amino acid sequences of tryptic fragments of TRAIL-R protein purified  
20 from Jurkat cells and from PS-1 cells (RX 1015, p. 18, ¶ 4 - p. 19, ¶ 1).

21 96. TRAIL-R protein purified from Jurkat cells and from PS-1 cells were both  
22 said to yield a tryptic fragment having the same amino acid sequence,  
23 i.e., VPANEGD (RX 1015, p. 19, ¶ 1).

- 1        97. Two other tryptic fragments obtained from TRAIL-R protein purified from  
2        PS-1 cells were said to have amino acid sequences of VCEC and  
3        SGEVELSSV, respectively (RX 1015, p. 19, ¶ 2).
- 4        98. Example 3 of the '255 specification is said to describe isolating and  
5        amplifying a TRAIL-R DNA fragment from a PS-1 cell cDNA (RX 1015.,  
6        p. 19, ¶ 3).
- 7        99. Figure 1 of the '255 application is said to show the nucleotide and  
8        encoded amino acids sequences of the isolated TRAIL-R DNA fragment:  
9        ETLRQCFDDFADLVPFDSWEPLMRKLGLMDNEIKVAKAEAAGHRDTLX  
10       TML (RX 1015, p. 19, p. 19, ¶ 3; p. 24).
- 11       100.     According to the '861 specification, TRAIL-R is a protein which  
12       binds TRAIL and, thus, finds uses in affinity chromatography purification  
13       of TRAIL and in inhibiting biological activities of TRAIL (RX 1014, II. 26-  
14       30)
- 15       101.     The '861 specification states that Example 1 discloses the isolation  
16       and purification of human TRAIL-R protein with a molecular weight of  
17       about 52 kD from the cell membranes of Jurkat cells (RX 1014, p. 15, II.  
18       27-34).
- 19       102.     Specifically, "Jurkat cells are disrupted, and the subsequent  
20       purification process includes affinity chromatography (employing a  
21       chromatography matrix containing TRAIL), and reversed phase HPLC"  
22       (RX 1014, p. 4, II. 5-7).

1           103.       Further according to the '861 specification, Example 2 discloses the  
2           amino acid sequences of tryptic fragments of TRAIL-R protein purified  
3           from Jurkat cells and from PS-1 cells (RX 1014, p. 18, ll. 7-31).

4           104.       TRAIL-R protein purified from Jurkat cells and from PS-1 cells were  
5           both said to yield a tryptic fragment having the same amino acid  
6           sequence, i.e., VPANEGD (RX 1014, p. 18, ll. 7-25).

7           105.       Two other tryptic fragments obtained from TRAIL-R protein purified  
8           from PS-1 cells were said to have amino acid sequences of VCEC and  
9           SGEVELSSV, respectively (RX 1014, p. 18, ll. 27-32).

10          Rauch acknowledges that, unlike the '852 and the '536 applications, neither  
11          the '255 nor the '861 applications disclose the full amino acid sequence of  
12          TRAIL-R as presented in SEQ ID NO:2 of the '358 patent (Paper 34, p. 5, ¶ 3  
13          and p. 7, ¶ 1). Rauch argues that (a) the isolated, purified TRAIL-R protein  
14          disclosed in the '255 and '861 applications inherently has an amino acid  
15          sequence at least 90% identical to that set forth in SEQ ID NO:2 of the '358  
16          patent and (b) the '255 and '861 applications disclose that TRAIL-R binds TRAIL  
17          (Paper 34, p. 8, ¶ 1 and ¶ bridging pp. 7-8). Rauch relies on the testimony of Dr.  
18          Cheng in support of its position.

19          106.       According to Dr. Cheng, the disclosure of the '861 application,  
20          specifically Examples 1 and 2, "would lead one of ordinary skill in the art  
21          to conclude that the inventors had possession of an isolated, purified  
22          protein that bound TRAIL at the time the '861 Application was filed" from  
23          the membranes of Jurkat cells, said protein having a molecular weight of

1           about 50-55 kD as determined by SDS-PAGE and a partial amino acid  
2           sequence of VPANEGD (RX 1039, ¶ 8).

3           107.       Further according to Dr. Cheng, the disclosure of the '255  
4           application is substantially the same as that of the '861 application and  
5           additionally discloses a 51 amino acid sequence bearing significant  
6           homology to the death domains found in TNF receptor proteins TNFR1  
7           and Fas (RX 1039, ¶ 10).

8           108.       Still further according to Dr. Cheng,  
9                    identification of a putative death domain in TRAIL-R,  
10                   combined with the experimental data previously  
11                   disclosed in the '861 Application showing the isolation  
12                   and purification of TRAIL-R, its molecular weight, and  
13                   its ability to bind TRAIL, would be sufficient to convey  
14                   to one of ordinary skill in the art that the inventors  
15                   were in possession of a TRAIL receptor belonging to  
16                   the TNFR family at the time the '255 Application was  
17                   filed in March of 1997 (RX 1039, ¶ 10).

18           109.       Dr. Cheng concluded that a skilled artisan would recognize that the  
19           isolated, purified TRAIL-R protein disclosed in the '861 and '255  
20           applications had an amino acid sequence which was later determined to  
21           be a TR-2 sequence which is at least 90% identical to the amino acid  
22           sequence set forth in Rauch SEQ ID NO:2 as required by Count 1 (RX  
23           1039, ¶¶ 8-9).

24           Ni contends that Rauch's inherency theory is flawed. Specifically, Ni argues  
25           that any TRAIL-R protein purified by the method disclosed in the respective  
26           Example 1 of the '861 and '255 specifications is necessarily the mature form of a  
27           TRAIL-R protein, which lacks its leader sequence (signal peptide) and, therefore,

1 would not have an amino acid sequence that is at least 90% identical to the  
2 amino acid sequence of SEQ ID NO:2 of the '358 patent. [Paper 47, p. 4, ¶ 2.]

3 110. TRAIL-R protein is expressed on the membranes of Jurkat cells  
4 (see e.g., RX 2137, p. 700, c. 2, ¶ 2).

5 111. The isolation and purification method disclosed in Example 1 of the  
6 '861 and '255 applications and of the '358 patent are essentially identical  
7 (compare Example 1 in each of RX 1015 (pp. 16-18), RX 1014 (pp. 15-  
8 17) and RX 1042 (cc. 23-25)).

9 112. Dr. Cheng also testified that Example 1 of the '861 application and  
10 the '358 patent are essentially identical, but for minor spelling  
11 differences, e.g., abbreviating California as "CA" in one and "Calif" in the  
12 other (NX 2124, p. 98, l. 3 - p. 99, l. 17).

13 113. According to Dr. Cheng, the method of Example 1 would yield  
14 mostly mature TRAIL-R protein because it was obtained from Jurkat cell  
15 membranes (NX 2124, p. 93, l. 13 - p. 95, l. 8; p. 101, ll. 3-7; p. 103, ll. 8-  
16 12; p. 104, l. 17- p. 105, l. 3).

17 114. According to the involved '358 patent, analysis of tryptic fragments  
18 obtained from a mature TRAIL-R protein shows that its N-terminal is  
19 amino acid residue 56 of SEQ ID NO:2, i.e., that a 55 amino acid  
20 signal peptide was cleaved off of full length TRAIL-R protein when  
21 TRAIL-R was inserted into the cell membrane (RX 1042, c. 3, ll. 1-32).

22 115. Dr. Cheng testified that a mature form of TRAIL-R protein having  
23 385 amino acid residues (i.e., missing its 55 amino acid leader

1           sequence) is 87.5 percent identical to 440 amino acid full length  
2           TRAIL-R protein (NX 2124, p. 95, I. 25 - p. 97, I. 23 and p. 115, II. 10-  
3           21 (dividing 385 by 440 and multiplying by 100 to yield %)).

4           116.   However, according to Dr. Cheng, simply dividing the number of  
5           identical residues in two proteins by the number of residues in the  
6           longer protein was neither the only way of determining percent identity  
7           between the proteins nor the preferred method (NX 2124, p. 108, II. 20-  
8           24).

9           117.   While the '358 patent specification does not define "percent  
10          identity" as that term is used in its claims, the '358 specification states  
11          that "percent identity may be determined, for example, by comparing  
12          sequence information using the GAP computer program, version 6.0  
13          described by Devereux et al. (*Nucl. Acids Res.* 12:387, 1984) (RX  
14          1042. c. 6, I. 65 - c. 7, I. 1).

15          118.   In Dr. Cheng's opinion, the mature and full length forms of TRAIL-R  
16          are the same protein because they are from the same gene (NX 2124,  
17          p. 101, II. 12-24).

18          119.   Neither Dr. Cheng nor Ni determined what percent identity a mature  
19          form of TRAIL-R protein having 385 amino acid residues would have to  
20          440 amino acid full length TRAIL-R protein set forth in SEQ ID NO:2 of  
21          the '358 patent using the GAP computer program, version 6.0  
22          described by Devereux et al. (*Nucl. Acids Res.* 12:387, 1984) set forth  
23          in the '358 patent (RX 1042. c. 6, I. 65 - c. 7, I. 1).



1       The count requires, in relevant part, an isolated TRAIL-R polypeptide  
2   having an amino acid sequence that is at least 90% identical to SEQ ID NO:2 of  
3   the '358 patent. Rauch contends that the isolated, purified TRAIL-R protein  
4   disclosed in the '255 and '861 applications inherently satisfies this limitation. As  
5   stated in In re Oelrich, 666 F.2d 578, 581, 212 USPQ 323, 326 (CCPA 1981)  
6   (quoting Hansgirk v. Kemmer, 102 F.2d 212, 214, 40 USPQ 665, 667 (CCPA  
7   1994)), "[i]nherence, however, may not be established by probability or  
8   possibilities. The mere fact that a certain thing may result from a given set of  
9   circumstances is not sufficient."

10       It is clear from Dr. Cheng's testimony that there are a number of ways of  
11   calculating percent identity between two given amino acid sequences, each of  
12   which may yield a different result. By at least one calculation, a TRAIL-R protein  
13   obtained from Jurkat cell membranes (as described in Rauch's '255 and '861  
14   applications) would be less than 90% identical to SEQ ID NO:2 of the '358 patent  
15   as required by Count 1 (i.e., Dr. Cheng calculated an 87.5 % identity (FF 115)).  
16   Using a different method may give a different result (e.g., Tartaglia calculated  
17   29% identity over 45 amino acids, but extended the region of homology an  
18   additional 20 amino acids by introducing a single amino acid gap in one of the  
19   sequences (FF 46)). While Dr. Cheng has stated that some methods of  
20   calculating percent identity are preferred over others, neither Dr. Cheng nor  
21   Rauch has pointed to evidence of record establishing an art recognized standard  
22   method of calculating percent identity between amino acid sequences.  
23   Furthermore, neither Dr. Cheng nor Rauch has pointed to an art recognized

1 method of calculation which establishes at least a 90% identity between the  
2 amino acid sequences. Additionally, software programs used to calculate  
3 percent identity are programmed to create different alignments based on different  
4 methods (FF 43). In short, not only do different methods of calculating percent  
5 identity give different results, but also apparently there is no standard method in  
6 the art for calculating percent identity. Thus, one method might yield a percent  
7 identity that falls within the count, while another method might not. Since the  
8 specification of the '358 patent does not define how to determine "percent  
9 identity" (FF 117), there is no defined method for determining whether a  
10 particular amino acid sequence is "at least 90% identical" to SEQ ID NO:2 of the  
11 '358 patent as required by the count. Moreover, to the extent the '358 patent  
12 suggests that the GAP computer program, version 6.0 described by Devereux et  
13 al. (*Nucl. Acids Res.* 12:387, 1984) might be the preferred method for  
14 determining percent identity between two sequences (FF 117), neither Ni, Rauch  
15 nor Dr. Cheng have shown that applying this calculation will result in at least 90%  
16 sequence identity between mature and full length TRAIL-R proteins. Therefore,  
17 Rauch has failed to establish that the isolated, purified TRAIL-R protein disclosed  
18 in the '255 and '861 applications inherently has an amino acid sequence at least  
19 90% identical to that set forth in SEQ ID NO:2 of the '358 patent. Consequently,  
20 Rauch substantive motion 1 is **denied** as to the '255 and '861 applications.

21 Ni's argument that the '255 and '863 applications fail to satisfy the how-to-  
22 use requirement of § 112, first paragraph, because the '255 and '863 applications  
23 allegedly fail to disclose any utility for the described TRAIL-R protein is not

1 persuasive in view of their respective disclosures (FFs 92 and 100) and  
2 Appendices D and C, respectively, attached to Paper 34 for substantially the  
3 same reasons set forth above in regard to the '536 and '852 applications. It is  
4 unnecessary to reach the merits of Ni's two remaining arguments based on  
5 molecular weight and alleged errors in amino acid sequences. In particular, we  
6 need not consider what effect errors in amino acid sequencing might have on the  
7 percent identity between the sequence containing some errors and SEQ ID NO:2  
8 of the '358 patent.

9 Based on the foregoing, Rauch substantive motion 1 is **granted** to the  
10 extent that Rauch is accorded benefit for purposes of priority of the 4 June 1997  
11 and 28 March 1997 filing dates of applications 08/869,852 and 08/829,536,  
12 respectively, and **otherwise denied**.

13 **IX. Ni Substantive Motion 3**

14 Pursuant to 37 CFR § 41.121(a)(1)(iii) and the Order issued 29 November  
15 2005 (Paper 26), Ni seeks judgment that all of Rauch's involved claims, claims 1,  
16 4-6, 8-11, 17-19, 26-28, 34, 37, 38 and 40, are unpatentable under 35 U.S.C.  
17 § 102(e) as anticipated by U.S. Patent 6,872,568 ("Ni's '568 patent," NX 2004)  
18 (Paper 31). Rauch opposes (Paper 54); Ni replies (Paper 62).

19 120. Ni's '568 patent issued from application 09/565,009 ("the '009  
20 application"), filed 4 May 2000 (NX 2004, title page (21), (22), (75)).

21 121. The '009 application is said to be a continuation-in-part of  
22 application 09/042,583 ("the '583 application," NX 2024), filed 17 March  
23 1998 (NX 2004, title page (63)).

1       122.       When the '583 application was filed, Ni claimed benefit under 35  
2       U.S.C. § 119(e) to provisional applications 60/040,846 (NX 2042) and  
3       60/054,021 (NX 2056), filed 17 March 1997 and 29 July 1997,  
4       respectively (NX 2024, p. 1, ll. 12-14).

5       123.       Rauch's involved '358 patent issued from the '392 application, filed  
6       25 May 2000 (FF 6), after the filing of the '009 application.

7       124.       According to the '392 application, the '392 application is

8       (i)       a divisional of the '036 application (RX 1018), filed 26 June 1997,

9       (ii)       a continuation-in-part of the '852 application (RX 1017), filed 4 June  
10       1997,

11       (iii)       a continuation-in-part of the '536 application (RX 1016), filed 28  
12       March 1997,

13       (iv)       a continuation-in-part of the '255 application (RX 1015), filed 12  
14       March 1997,

15       (v)       a continuation-in-part of the '861 application (RX 1014), filed 13  
16       February 1997 (RX 1012, title sheet (60)).

17       Ni contends that Rauch's involved '358 patent claims are unpatentable under  
18       35 U.S.C. § 102(e) based on Ni's '568 patent (Paper 31, p. 2, ¶ 4). Ni's '568  
19       patent issued from an application filed three weeks before Rauch's application  
20       which issued as the '358 patent was filed (FFs 120 and 123). Therefore, on its  
21       face, the '568 patent is prior art to Rauch's involved claims. However, both Ni  
22       and Rauch assert that their respective '568 patent reference and involved claims  
23       are entitled to benefit of the filing dates of a number of earlier applications (FFs

1 121, 122 and 124). Specifically, Ni argues that Rauch's claims are not entitled to  
2 a priority date any earlier than the 28 March 1997 filing date of Rauch's '536  
3 application, while Ni's '568 patent is entitled to the 17 March 1997 filing date of its  
4 '846 application (Paper 31, p. 8, ¶ 1 and p. 11, ¶ 3). Therefore, before deciding  
5 whether the disclosure of Ni's '568 patent anticipates the subject matter of  
6 Rauch's claims, we must first decide, as a matter of law, whether Ni's '568 patent  
7 and Rauch's '358 patent are entitled to the filing date of one or more of the  
8 applications to which they have claimed priority.

9 For prior art purposes, a patent is entitled to benefit of the filing date of a  
10 parent application as to all subject matter carried over into the patent from the  
11 parent application when the parent application discloses the invention claimed in  
12 the reference patent pursuant to 35 U.S.C. § 120 (and related statutes). In re  
13 Wertheim, 646 F.2d 527, 539, 209 USPQ 554, 565-66 (CCPA 1981). According  
14 to § 120, a subsequent application is permitted to relate back to the filing date of  
15 a prior application disclosing the same invention if the subsequent application is  
16 for an invention disclosed in the manner provided by the first paragraph of 35  
17 U.S.C. § 112, is submitted by the same inventor, is filed before the abandonment  
18 of the first application and specifically refers to the parent application. To satisfy  
19 the requirements of § 112, there must be a written description and an enabling  
20 disclosure of the full scope of the claimed subject matter. Warner-Lambert Co.,  
21 v. Teva Pharmaceuticals USA, Inc., 418 F.3d 1326, 1336-37, 75 USPQ2d 1865,  
22 1871-72 (Fed. Cir. 2005) (full scope of claims must be enabled); Pandrol USA,  
23 LP v. Airboss Railway Products, Inc., 424 F.3d 1161, 1165, 76 USPQ2d 1524,

1 1526 (Fed. Cir. 2005) (written description must show possession of the claimed  
2 invention). Moreover, to get the benefit of the filing date of an earlier application  
3 under § 120 (and related statutes) where there is a chain of applications, there  
4 must be a chain of copending applications each of which satisfies the  
5 requirements of § 112, first paragraph, for the claimed subject matter. In re  
6 Hogan, 559 F.2d 595, 609, 194 USPQ 527, 540 (CCPA 1977). Thus, to the  
7 extent that a continuation-in-part application adds new matter, claims that are  
8 dependent upon the new matter are only entitled to the filing date of the  
9 continuation-in-part application and not that of the parent application.

10 Ni's '568 patent issued from a continuation-in-part application (the '009  
11 application) of parent application 09/042,583 (FFs 120 and 121) which claimed  
12 § 119(e) benefit of two provisional applications, 60/054,021 and 60/040,846 (FF  
13 122). Thus, in order for Ni's '568 patent to qualify as prior art § 102(e)(2) as of  
14 the 17 March 1997 filing date of its '846 application, Ni must show (1) that the  
15 subject matter claimed in the '568 patent was disclosed in the '583 parent  
16 application and in the '846 provisional application and (2) that the subject matter  
17 relied upon in the '846 provisional application was carried forward into the '583  
18 parent application and into the '568 patent.

19 Ni fails to make either showing.

20 125. The '568 patent claims isolated antibodies or fragments thereof that  
21 specifically bind to a protein "consisting of amino acid residues 1 to 133  
22 of SEQ ID NO:2" or to "the extracellular domain of the protein encoded  
23 by the cDNA contained in ATCC Deposit No. 97920," isolated cells and

1           hybridomas producing said antibodies/fragments, and methods of  
2           detecting DR5 using said antibodies/fragments (NX 2004, cc. 157-162).

3           Ni has neither argued nor pointed out where the antibody-based subject  
4           matter claimed in the '568 patent is disclosed in the '583 parent application or in  
5           either the '021 or '846 provisional application. The '568 patent was based on a  
6           continuation-in-part application and, therefore, presumptively contains additional  
7           and/or different subject matter than the '583 parent application. Ni has neither  
8           argued nor pointed out where the subject matter of either provisional application  
9           relied upon was carried forward into the '583 parent application and into the '568  
10          patent.

11          Ni simply asserts that the '568 patent issued from the '009 application which  
12          claimed priority as a continuation-in-part of the '583 application which claimed  
13          priority to the '021 and '846 provisional applications (Paper 31, ¶ 2). According to  
14          Ni, the '846 application "contains the entire nucleic acid sequence and the  
15          polypeptide sequence encoded thereby of a human DR5 protein" (Paper 31, p. 9,  
16          ¶ 1 (citations to SMF omitted)). The main focus of Ni's arguments is on  
17          disclosure in the '846 provisional application alleged to disclose the subject  
18          matter of Rauch's involved claims. There is little, if any, discussion of the claims  
19          of the '583 parent application and no argument or assertion that either the '583 or  
20          '846 application provides § 112, first paragraph, support for the claimed subject  
21          matter of the '568 patent. Thus, Ni has failed as matter of law to establish prima  
22          facie that its '568 patent is entitled to the filing date of the '583 parent application  
23          or either the '021 or '846 provisional application. Consequently, based on this

1 record, the '568 patent has only been shown to be entitled to a filing date of 4  
2 May 2000 for prior art purposes.

3       Additionally, we do not see how the filing date of either the '021 or '846  
4 provisional applications can be accorded to the '568 patent as its § 102(e) filing  
5 date. First, provisional applications were established to place domestic  
6 applicants on equal footing with foreign applicants with respect to rights of  
7 priority. 35 U.S.C. § 119(e). Section 102(e) of title 35 provides, in relevant part,  
8 that "A person shall be entitled to a patent unless ... (e) the invention was  
9 described in ... (2) a patent granted on an application for patent by another filed  
10 in the United States before the invention by the applicant for patent ...". Here, the  
11 reference being relied upon to show unpatentability under § 102(e) is the '568  
12 patent, not the '021 or '846 provisional application. Second, in reaching its  
13 conclusion in Wertheim<sup>14</sup> that a subsequent application is permitted to relate  
14 back to the filing date of a prior application disclosing the same invention if the  
15 subsequent application is for an invention disclosed in the manner provided by  
16 the first paragraph of 35 U.S.C. § 112, the CCPA stated:

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<sup>14</sup> In Wertheim, the examiner made a 35 U.S.C. § 103 rejection over a U.S. patent to Pfluger. The Pfluger patent (Pfluger IV) was the child of a string of abandoned parent applications (Pfluger I, the first application, Pfluger II and III, both continuations-in-part). Pfluger IV was a continuation of Pfluger III. The court characterized the contents of the applications as follows: Pfluger I - subject matter A; Pfluger II - subject matter AB; Pfluger III, subject matter ABC; and, Pfluger IV - subject matter ABC. ABC anticipated the claims of the application being examined, but the filing date of Pfluger III was later than the application filing date. The Examiner reached back to subject matter A in Pfluger I and combined this disclosure with another reference to establish obviousness under § 103. The court held that the Examiner impermissibly carried over subject matter A and should have instead determined which of the parent applications contained the subject matter which made Pfluger patentable. Only if subject matter B and C were not claimed, or at least not critical to the patentability of Pfluger IV could Pfluger IV rely on the filing date of Pfluger I. The court determined that Pfluger IV was only entitled to the filing date of Pfluger III and reversed the rejection, noting that the added new matter C was critical to the claims of the issued patent.



1           The dictum in Lund, supra, that

2                   \* \* \* the continuation-in-part application is  
3                   entitled to the filing date of the parent  
4                   application as to all subject matter *carried over*  
5                   into it from the parent application \* \* \* for  
6                   purposes of \* \* \* utilizing the *patent* disclosure  
7                   as evidence to defeat another's right to a  
8                   patent \* \* \* [emphasis in the original]

9           is hereby modified to further include the requirement  
10           that the application, the filing date of which is needed  
11           to make a rejection must disclose, pursuant to  
12           §§ 120/112, the invention claimed in the reference  
13           patent. Where continuation-in-part applications are  
14           involved, the logic of the Milburn holding as to secret  
15           prior art would otherwise be inapplicable. Without the  
16           presence of a patentable invention, no patent could  
17           issue "but for the delays of" the PTO.

18   Wertheim, 646 F.2d at 539, 209 USPQ at 565-66. Here, Ni has not shown that  
19   the subject matter claimed in the '568 patent could have issued earlier "but for  
20   the delays" of the PTO and, therefore, the '568 patent was entitled, as a matter of  
21   law, to the filing of either provisional application as its § 102(e) filing date. No  
22   U.S. patent can issue from a provisional application filed under § 111(b).  
23   Therefore, any time a provisional application is pending is not a delay that can be  
24   attributed to the PTO under the Milburn delay theory. Again, Ni has failed as  
25   matter of law to establish prima facie that its '568 patent is entitled to the filing  
26   date of either the '021 or '846 provisional application.

27       Rauch, on the other hand, appears to have confused benefit accorded for  
28   purpose of priority in an interference contest with benefit accorded under § 120  
29   (see e.g., Paper 54, p. 17). Nonetheless, Rauch has provided detailed claim  
30   charts said to show where the claimed subject matter of Rauch's involved '358  
31   patent is supported by each of its asserted priority applications (Paper 54,

1 Appendices D through H). For example, Rauch asserts that Appendix H (Paper  
2 54, pp. 143-166) describes where the '036 parent application, said to be a  
3 divisional of the application from which Rauch's involved '358 patent issued (FFs  
4 122 and 123), provides support for each claim of Rauch's involved '358 patent on  
5 a claim-by-claim basis. Based on the evidence submitted, Rauch has prima facie  
6 established that its involved claims are at least entitled to benefit of the 26 June  
7 1997 filing date of its '036 parent application. Ni does not dispute Rauch's claim  
8 to benefit of the filing date of the '036 application (Paper 31, p. 8, ¶ 1 and p. 11, ¶  
9 3).

10 In summary, since the '568 patent has only been shown to be entitled to a  
11 filing date of 4 May 2000 for prior art purposes and Rauch's involved claims have  
12 been shown to be entitled to a filing date of at least 26 June 1997, the '568 patent  
13 does not qualify as prior art vis-à-vis Rauch's involved claims under § 102(e). It  
14 is not necessary to consider whether Rauch's involved claims are entitled to one  
15 or more of the filing dates of Rauch's still earlier filed '852, '536, '255 or '861  
16 application. Moreover, since the '568 patent has not been shown to be prior art  
17 under § 102(e), it is not necessary for us to consider the content of the '568  
18 patent.

19 Based on the foregoing, Ni substantive motion 3 is **denied**.

20 **X. Rauch Miscellaneous Motion 5**

21 Pursuant to 37 CFR § 41.155(c), Rauch seeks to exclude selected  
22 portions of the direct testimony of Dr. Reed that reference a person of ordinary  
23 skill in the art from evidence (NX 2103, ¶¶ 16, 19, 21-28, 30-43, 45-48, 50-52, 56

1 and 63-64), contending that his definition of ordinary skill "is so broad that it fails  
2 to limit 'one of ordinary skill in the art' to any substantive or realistic meaning of  
3 such person" (Paper 76, p. 5, ¶ 2). Thus, Rauch argues, any statement by Dr.  
4 Reed regarding what one of ordinary skill in the art would have known or  
5 understood in 1997 is irrelevant, lacking foundation, prejudicial and confusing  
6 (Paper 76, p. 6, ¶ 1). Rauch further seeks to exclude selected portions of the  
7 redirect testimony of Dr. Reed from evidence as improper redirect, leading and  
8 prejudicial (NX 2123, p. 169, ll. 2-21 and p. 172, l. 16 - p. 173, l. 13) (Paper 76, p.  
9 9, ¶ 2; pp. 11-12). Finally, Rauch seeks to exclude selected portions of the direct  
10 testimony of Dr. Andrew Badley (NX 2157, ¶¶ 26-27, 31-32 and 34-38), also  
11 contending that Dr. Badley's definition of a "person of ordinary skill in the art" is  
12 so flawed that any statement by Dr. Badley regarding what one of ordinary skill in  
13 the art would have known or understood in 1997 is irrelevant, lacking foundation,  
14 prejudicial and confusing (Paper 76, p. 13, ¶ 2 and p. 15, ¶ 1). Rauch further  
15 contends that Dr. Badley lacks sufficient expertise on the subject matter of his  
16 testimony (Paper 76, p. 16, ¶ 2).

17 126. Rauch timely filed its objections to the evidence sought to be  
18 excluded (RXs 1094 and 1095; NX 2123, p. 161; p. 166, l. 2; p. 169, ll. 10  
19 and 16; p. 172, l. 20; p. 173, ll. 7-8).

20 Rauch identifies the objected to testimony of Dr. Reed as submitted in  
21 support of Ni substantive motion 2, Ni reply 2, Ni reply 3 and Ni opposition 3 to  
22 Rauch substantive motion 3 (Paper 76, Appendix D). First, Rauch's arguments  
23 go to the weight to be accorded Dr. Reed's testimony based on the

1 reasonableness of his conclusions as assessed by one of ordinary skill in the art  
2 in view of the state of the art at the relevant time, not to its admissibility. Second,  
3 having considered the testimony of both Dr. Reed and Dr. Cheng, we credited  
4 the testimony of the latter over that of the former as discussed in our denial of the  
5 relevant portion of Ni substantive motions 2 and 3 and in our grant of the relevant  
6 portion of Rauch substantive motion 3. Therefore, Rauch substantive motion 5 is  
7 dismissed as moot to the extent it seeks to exclude selected portions of the direct  
8 and redirect testimony of Dr. Reed since we have not relied upon either the direct  
9 or redirect testimony of Dr. Reed to Rauch's detriment.

10 Rauch identifies the objected to testimony of Dr. Badley as submitted in  
11 support of Ni opposition 1 to Rauch substantive motion 1, Ni opposition 3 to  
12 Rauch substantive motion 3, Ni opposition 4 to Rauch substantive motion 4 and  
13 Ni reply 3. Again, Rauch's arguments go to the weight to be accorded Dr.  
14 Badley's testimony based on the reasonableness of his conclusions as assessed  
15 by one of ordinary skill in the art in view of the state of the art at the relevant time,  
16 not to its admissibility. Since Rauch substantive motion 4 was dismissed as  
17 moot, we did not reach Ni opposition 4 thereto. Furthermore, since Ni did not  
18 meet its burden of proof as discussed in our denial of Ni substantive motion 3, we  
19 did not reach Ni reply 3. Similarly, as discussed in our denial of the relevant  
20 portions of Rauch substantive motions 1 and 3, since Rauch did not meet its  
21 burden of proof as movant, we did not reach Ni oppositions 1 and 3 thereto.  
22 Likewise, as discussed in our granting of the relevant portions of Rauch

1 substantive motions 1 and 3, we credited the testimony of Dr. Cheng and did not  
2 rely upon the direct testimony of Dr. Badley to Rauch's detriment.

3 Based on the foregoing, Rauch substantive motion 5 is **dismissed** as  
4 moot since we have not relied upon any of the objected to testimony sought to be  
5 excluded to Rauch's detriment.

6 **XI. Ni Miscellaneous Motion 4**

7 Pursuant to 37 CFR § 1.155(c), Ni seeks to exclude from evidence:

8 (a) exhibits related to Rauch's priority statements in (i) related interference  
9 105,240 (RX 1074), (ii) this interference (RX 1025, RX 1038, RX 1052 and RX  
10 1054)<sup>15</sup> and (iii) related interference 105,380 (RX 1051);

11 (b) direct (RX 1074) and deposition (NX 2179-2181) testimony of Dr. Gavin  
12 R. Screanton in related interference 105,240;

13 (c) direct testimony of Norman Boiani (RX 1075); and,

14 (d) selected portions of the redirect testimony of Dr. Cheng (NX 2124, p.  
15 132, l. 16 - p. 135, l. 5 and p. 135, l. 9 - p. 136, l. 13) (Paper 86, pp. 1-2). Rauch  
16 opposes (Paper 80); Ni replies (Paper 88).

17 Ni contends (Paper 86, pp. 22-23) that

18 RX 1025, RX 1038, RX 1051, RX 1052 and RX 1054  
19 should be excluded under FRE 901 for lack of  
20 authentication and lack of foundation. In addition,  
21 these exhibits should be excluded under FRE 1001  
22 (4), 1002, and 1003, *inter alia*, because none of these  
23 exhibits appear to be originals nor admissible  
24 duplicates of the originals. Furthermore, these  
25 exhibits should be excluded under FRE 403, *inter alia*,  
26 because its [sic] probative value, if any, is outweighed

---

<sup>15</sup> Exhibits RX 1025 and RX 1038 are also relied upon in Rauch's priority statement in related interference 105,240.

1 by considerations of waste of time, lack of  
2 authentication and the reliability of the copies.

3 Furthermore, RX 1074, the declaration of Dr.  
4 Gavin R. Screaton, should be excluded under FRE  
5 403 because its probative value, if any, is far  
6 outweighed by confusion of the issues. In addition,  
7 RX 1074 should be excluded under 37 C.F.R.  
8 § 41.122(b) because the declaration does not  
9 respond to arguments raised in an opposition but  
10 merely is an attempt by Rauch to make additional  
11 arguments in a reply that should have been raised in  
12 a motion. Furthermore, contingent upon the Board  
13 excluding RX 1074, Party Ni moves to exclude NX  
14 2179, NX 2180 and NX 2181 for being irrelevant  
15 under FRE 401 and confusing the issues under FRE  
16 403.

17 In addition, Party Ni moves to exclude RX  
18 1075, the Declaration of Norman Boiani, under FRE  
19 1002 because Exhibit A appears to be a photocopy,  
20 not an original, of a laboratory notebook page.  
21 Furthermore, Party Ni moves to exclude RX 1075  
22 under FRE 403 because Exhibit A of RX 1075 is  
23 taken out of context of the rest of the laboratory  
24 notebook. Party Ni's inability to determine the context  
25 of Exhibit A is unfairly prejudicial and this prejudice far  
26 outweighs any probative value of RX 1075.

27 Lastly, the above-cited portions of NX 2124  
28 should be excluded under FRE 611(c), FRE 403, and  
29 Cross Examination Guideline [3] of the Standing  
30 Order. The leading questions asked by Rauch's  
31 counsel clearly suggested single answers to the  
32 witness which resulted in the interjection of the  
33 opinions of counsel for Rauch in place of Dr. Cheng's  
34 opinions. Clearly the prejudicial effect of such  
35 testimony far outweighs its probative value, and the  
36 above-cited evidence should be excluded or, at most,  
37 accorded little weight by the Board.

38 Ni's motion has serious procedural defects. Rule 155(c) provides that a  
39 motion to exclude evidence must explain the objections and identify the  
40 objections in the record. As explained in Standing Order ¶ 21.3(a) a motion to

1 exclude evidence shall (1) identify where in the record the objection was  
2 originally made and (2) identify where in the record the evidence was relied upon  
3 by the opponent, and (3) address objections to exhibits (in whole or in part) in  
4 exhibit numerical order. According to Standing Order § 21.1, the objection to the  
5 admissibility of evidence should be filed as part of a motion to exclude the  
6 evidence.

7 First, Ni contends that it timely objected to exhibits RX 1025, RX 1038, RX  
8 1051, RX 1052 and RX 1054 as shown in exhibits NX 2194 and NX 2195, filed in  
9 support of its motion.

10 127. Ni exhibits NX 2194 and NX 2195 are "REDACTED" papers entitled  
11 "NI OBJECTIONS TO THE ADMISSIBILITY OF RAUCH'S SUPPLEMENT  
12 EXHIBIT 1054 AND RAUCH'S RESPONSES TO NI'S OBJECTIONS TO  
13 EXHIBITS AND 1050-1052" and "NI OBJECTIONS TO THE  
14 ADMISSIBILITY OF RAUCH EXHIBITS 1050, 1051 AND 1052,"  
15 respectively.

16 128. Ni has not provided evidence that it timely objected to exhibits RX  
17 1025 and RX 1038.

18 129. Ni has not identified where in the record exhibits RX 1025, RX  
19 1038, RX 1051, RX 1052, RX 1054 and RX 1075 were relied upon by  
20 Rauch.

21 130. According to Ni, RX 1074 and NX 2124 were relied upon in Rauch  
22 replies 1, 3 and 4 (Paper 86, p. 6, ¶ 3 and p. 7, ¶ 1).

1       131.       Rauch's exhibit list (Paper 93, p. 7) identifies exhibit RX 1051 as a  
2       document upon which Rauch will rely to prove its earliest corroborated  
3       conception of the subject matter of the count in related interference  
4       105,380.

5       132.       Similarly, Rauch's exhibit list (Paper 93, p. 10) identifies exhibit RX  
6       1074 as the declaration of Dr. Gavin R. Screanton filed in related  
7       interference 105,240.

8       Thus, the deposition testimony of Dr. Screanton (NX 2179-2181) is part of  
9       related interference 105,240, not this interference. Indeed, Ni's motion to  
10      exclude NX 2179-2181 is expressly contingent upon the Board excluding Dr.  
11      Screanton's direct testimony (RX 1074) (Paper 86, p. 22).

12      133.       Ni admits that Rauch has not relied on any testimony from Norman  
13      Boiani to date in this interference (Paper 86, p. 6, ¶ 4).

14      Thus, Ni has failed to object timely to evidence it seeks to exclude (RX 1025  
15      and RX 1038). Furthermore, Ni is seeking to exclude evidence which is either  
16      not of record in this interference (RX 1051, RX 1074, NX 2179-2181 and RX  
17      1075<sup>16</sup>) and/or has not been relied upon by Rauch in this interference (RX 1075).  
18      Therefore, Ni miscellaneous motion 4 to exclude evidence is **denied** as to  
19      exhibits RX 1025, RX 1038, RX 1051, RX 1074, NX 2179-2181 and RX 1075.

20      134.       Exhibits RX 1025, RX 1038, RX 1052 and RX 1054 are identified  
21      as documents said to prove Rauch's earliest corroborated date of

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<sup>16</sup> Rauch's Exhibit List explicitly states that exhibit RX 1075 is "WITHHELD" in this interference (Paper 93, p. 10, original emphasis).



1 conception of the subject matter of the count in this interference (Paper  
2 93, pp. 4, 5 and 7).

3 According to 37 CFR § 41.204(a)(2)(iv), a party filing a priority statement  
4 must "[p]rovide a copy of the earliest document upon which the party will rely to  
5 show conception." Exhibits RX 1025, RX 1038, RX 1052 and RX 1054 were  
6 served by Rauch in fulfillment of the requirement (FF 133). Ni does not contend  
7 that Rauch has relied on any of exhibits RX 1025, RX 1038, RX 1052 and RX  
8 1054 in support of any of Rauch's motion/opposition/reply papers. The time for  
9 Rauch to lay a foundation for and authenticate its exhibits RX 1025, RX 1038, RX  
10 1052 and RX 1054 is when Rauch relies upon them, i.e., as part of its priority  
11 motion. The time for us to weigh the reliability and probative value of exhibits RX  
12 1025, RX 1038, RX 1052 and RX 1054 is when they are submitted as evidence  
13 as part of Rauch's priority motion when the priority motion is filed. Therefore, Ni  
14 miscellaneous motion 4 to exclude evidence is **denied** as to exhibits RX 1025,  
15 RX 1038, RX 1052 and RX 1054.

16 As to the last evidence at issue, selected portions of the deposition  
17 testimony of Dr. Cheng (NX 2124, p. 132, l. 16 - p. 135, l. 5 and p. 135, l. 9 - p.  
18 136, l. 13), Ni contends that Rauch relied on the deposition testimony of Dr.  
19 Cheng in Rauch replies 1, 3 and 4 (Paper 86, p. 7, ¶ 1).

20 135. Ni explicitly directs our attention (Paper 86, pp. 17-18) to the  
21 following testimony as an example of how the redirect testimony of Dr.  
22 Cheng violates FRE 611(c), FRE 403 and Cross Examination Guideline  
23 [3]:

1 MR. WISE: Okay. Back on the record.

2 Q. I want to have you focus on paragraph 10.  
3 Paragraph 10 you said, "The specification of the '861  
4 application also contains additional substantial  
5 disclosure regarding antibodies to TRAIL-R, including  
6 methods for obtaining these antibodies and methods  
7 of obtaining antigen binding fragments of these  
8 antibodies."

9 And it says "'861 application, page 13, line 14 to page  
10 15, line 6."

11 Where in the specification of the '861 application  
12 would you find additional substantial disclosure  
13 relating to the antibodies for TRAIL-R?

14 A. You mean where I can find the information?

15 Q. Yes.

16 A. That's indicated here is the page 13 and the line  
17 14 to 15, line 14 through page 15 of line 6.

18 Q. Okay. Can you direct me to that, please.

19 A. Where is the --

20 Q. You have that there. You were looking at the  
21 claims and you were going to show me support and  
22 specification.

23 MR. GOLDSTEIN: Objection.

24 THE WITNESS: So it's indeed in the page is 13,  
25 there is a title, "Antibodies" section, and talking about  
26 how antibody generated, including the monoclonal  
27 and polyclone antibodies.

28 MR. GOLDSTEIN: I am going to move to strike the  
29 question and the answer.

30 First, since Rauch responsive motion 4 was dismissed as moot, we did not  
31 reach Rauch reply 4. Second, Ni did not explain where and how Rauch relied  
32 upon the objected to portions of Dr. Cheng's redirect testimony in Rauch replies 1

1 and 3 to support its position. For example, how did Rauch rely upon this  
2 allegedly elicited testimony to support its motion 1 for benefit of the filing date of  
3 an earlier application for the subject matter of a count directed to a genus of  
4 functional proteins, i.e., purified TRAIL-R polypeptides having an amino acid  
5 sequence that is at least 90% identical to SEQ ID NO:2 of Rauch's involved '358  
6 patent, wherein the polypeptides bind TRAIL. Third, to the extent Ni argues that  
7 the objected to portions of Dr. Cheng's redirect testimony are irrelevant,  
8 confusing or prejudicial, that objection goes to the weight to be accorded the  
9 testimony, not its admissibility. We have accorded Dr. Cheng's testimony the  
10 weight appropriate to its relevance and the underlying facts and data relied upon  
11 in support of his opinion. Ni has not shown otherwise. Therefore, Ni  
12 miscellaneous motion 4 to exclude evidence is **denied** as to the selected  
13 portions of the redirect deposition testimony of Dr. Cheng (NX 2124, p. 132, l. 16  
14 - p. 135, l. 5 and p. 135, l. 9 - p. 136, l. 13).

15 Based on the foregoing, Ni miscellaneous motion 5 is **denied**.

16 **XII. Order**

17 Based on the foregoing and for the reasons given, it is

18 ORDERED that Ni substantive motion 1 to substitute Ni proposed count 2  
19 for current Count 1 is **denied**;

20 FURTHER ORDERED that Ni substantive motion 2 for benefit for the  
21 purpose of priority is **dismissed** as moot as to Ni proposed count 2, **granted** as  
22 to the 29 July 1997 filing date of the 60/054,021 application for Count 1 and  
23 otherwise **denied**;

1           FURTHER ORDERED that Ni substantive motion 3 seeking judgment that  
2   all Rauch's involved claims are unpatentable under 35 U.S.C. § 102(e) as  
3   anticipated by U.S. Patent 6,872,568 is **denied**;

4           FURTHER ORDERED that Ni miscellaneous motion 4 to exclude certain  
5   evidence is **denied**;

6           FURTHER ORDERED that Rauch substantive motion 1 for benefit for the  
7   purpose of priority as to Count 1 is **granted** as to the 28 March 1997 and 4 June  
8   1997 filing dates of applications 08/829,536 and 08/869,852, respectively, and  
9   otherwise **denied**;

10          FURTHER ORDERED that Rauch substantive motion 2 to designate Ni  
11   claims 46, 55, 63, 64, 110 and 118 as corresponding to Count 1 is **denied**;

12          FURTHER ORDERED that Rauch substantive motion 3 is **granted** to the  
13   extent that Ni claims 35, 36, 38-45, 47-54, 56-61, 75, 83, 92, 99, 100, 102-109,  
14   111-116, 127-133, 168-178 and 180-203 are unpatentable under 35 U.S.C.  
15   § 102(e) as anticipated by U.S. Patent 6,072,047, **moot** as to anticipation under  
16   § 102(e) by U.S. Patents 6,642,358 and 6,569,642, and otherwise **denied**;

17          FURTHER ORDERED that Rauch responsive motion 4 is **dismissed** as  
18   moot in view of the denial of Ni substantive motion 1; and,

19          FURTHER ORDERED that Rauch miscellaneous motion 5 to exclude  
20   certain evidence is **dismissed** as moot.

**CAROL A. SPIEGEL**  
Administrative Patent Judge

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# Molecular Biology and Biotechnology

## A Comprehensive Desk Reference

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Sequences of nucleic acids in DNA and RNA and of amino acids in proteins define the primary structure of these molecules. Sequence analysis is carried out using computer programs that implement algorithms to determine sequence properties and to compare sequences. Sequence comparison can indicate whether an RNA or protein molecule or region of DNA is already known (identity) or has some degree of similarity to a known sequence. Sequence similarity may indicate similar structure or function. Sequence analysis can suggest the function of an unknown sequence based on the features it contains. Sequence analysis is a necessary preliminary to detailed experimental studies of structure, function, and interactions of biological macromolecules. Sequences are the information repository of the cell and a natural index to our growing understanding of cellular processes as dynamic systems of interactions between macromolecules.

## 1 PURPOSE OF SEQUENCE ANALYSIS

### 1.1 PREDICTION OF FUNCTION

Sequences that are unlike any known sequence may still be made to yield information that can suggest their possible function. The function of nucleic acids and proteins depends on their structure and involves complex interactions in three dimensions. It is not presently understood whether it is possible, in general, to derive structure from sequence. Sequence alone is therefore often inadequate to determine function. Predictions made from sequence analysis need to be experimentally tested. Nevertheless, computer analysis of sequences is valuable in suggesting the most useful experiments to perform.

### 1.2 REVEALING SIMILARITY

The first thing to do with a newly determined sequence is to compare it with all known sequences. The outcome may show identity to a known sequence, which may prove disappointing if one is hoping for something new. Similarity to a known sequence may suggest something new that can be characterized with relatively little effort. A totally unknown sequence may be a frustrating result: considerable effort will be needed to understand its function.

Sequence comparison is a nontrivial pursuit, and both statistical and biological considerations are involved. Statistically significant similarities (under some model and at some chosen level of significance) may be biologically meaningless. Sequence motifs that are statistically nonsignificant in similarity may encode the same function (this is likely to occur because the statistical model based on sequence alone is incomplete). In an area fraught with such difficulties, common sense and interpretation based on utility are paramount.

Sequence dissimilarity can range from identity, difference due to sequencing errors, difference due to population polymorphism (individual variants), and differences in multiple copies of a gene in a single individual (multigene families) to wide evolutionary divergence of genes in different organisms. Sequences that are similar due to common function may not share a common ancestral sequence in biological evolution. In general, ideas about the evolutionary relationships of sequences are not experimentally testable. Sequence homology (similarity due to descent from a common ancestor) is a hypothesis, not an observable fact, except in the case of microbial populations with high mutation rates and short

generation times, which may be studied experimentally through time.

## 2 ANALYSIS OF SINGLE SEQUENCES

### 2.1 DNA COMPOSITION, ISOCHORES, AND CODON USAGE

Nucleotides in DNA sequences may be counted as singlets, doublets, or triplets in either strand. Doublets or triplets may be counted as overlapping or nonoverlapping in two or three phases, respectively, on either strand. The genomes of various organisms vary considerably in their DNA composition. Warm-blooded vertebrates have a higher G+C content, which correlates with the higher thermal stability of GC over AT base pairs. Composition of regions within a genome can also vary considerably. Mammalian genomes contain relatively GC-rich and AT-rich regions, which are called isochores. Overlapping doublet frequencies are highly characteristic for an organism. CG dinucleotides are less common than expected in vertebrates and angiosperms, probably because spontaneous deamination of 5-methylcytosine to thymine prevents the repair of methylated CpG. In DNA coding for protein, one phase of nonoverlapping triplets will be the phase of translation and the triplets will be codons. In a gene, the possible codons for each amino acid are unevenly used, and the frequency table for the 64 triplets is called codon usage. Codon usage is different between different species and between highly and lowly expressed gene in the same species.

### 2.2 MAPPING DNA SEQUENCE FEATURES

Mapping the position of features on a DNA sequence is an important step in investigating its function. It is easy to map sites that can be precisely defined, such as stop codons or restriction enzyme recognition sites. Once DNA has been sequenced, the sizes of the fragments produced with any enzyme can be readily calculated. Features such as promoters, splice junctions, and ribosome binding sites are very difficult to predict because they are hard to specify. Mapping is most simply achieved by comparing the probe sequence with each position of the DNA sequence in turn and noting the hits. More sophisticated algorithms exist for rapid searching in large problems.

### 2.3 REPETITIVE SEQUENCES

Direct repeats and inverted repeats (sometimes called dyad symmetries) are common in DNA from many sources. Mammalian genomes contain families of long (LINE) and short (SINE) repeats. Repeats of *L1* (*Kpn* I) type are 5000 to 7000 bp long and are present in the genome in  $10^3$  to  $10^4$  copies. Repeats of *Alu* type are 350 bp long and occur in as many as  $9 \times 10^4$  copies. *Alu* repeats make human DNA hard to assemble from gel sequencing reads into the finished sequence. Inverted repeats occur in DNA coding for structural RNA, and these symmetry properties enable the RNA to fold into its secondary structure.

The dot plot is a diagram that reveals the presence of repeats and inverted repeats in sequences. It is also useful for comparing two different nucleic acid or protein sequences to detect regions of similarity. The dot plot is a rectangular array with rows labeled by one sequence and columns labeled by the other. A cell  $i, j$  can be used to represent the result of comparison of the  $j$ th residue of sequence A with the  $i$ th residue of sequence B. The simplest form of dot plot results from placing a diagonal mark in each cell where

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ent amino acids in proteins. Thus a 100-unit protein has  $20^{100}$  (more than  $10^{130}$ ) possible structures. This enormous variability means that cells and organisms can differ greatly in structure and function even though they are constructed of the same types of biopolymers produced by similar chemical reactions.

Starch (a storage form of glucose in plant cells), cellulose (a constituent of plant cell walls), and glycogen (a storage form of glucose in liver and muscle cells) are examples of another important type of biopolymer: the polysaccharide, which is built of sugar monomers (Figure 2-1). At least 15 different monomeric sugars can be bonded in multiple ways to form various polysaccharides; thus many polysaccharides are nonlinear, branched molecules.

Monomers are not the only small molecules important to cell structure. The lipids, for example, form the basic structure of cell membranes. Lipids cohere noncovalently in very large sheetlike complexes; the membranes thus formed are as crucial to living systems as are the biopolymers.

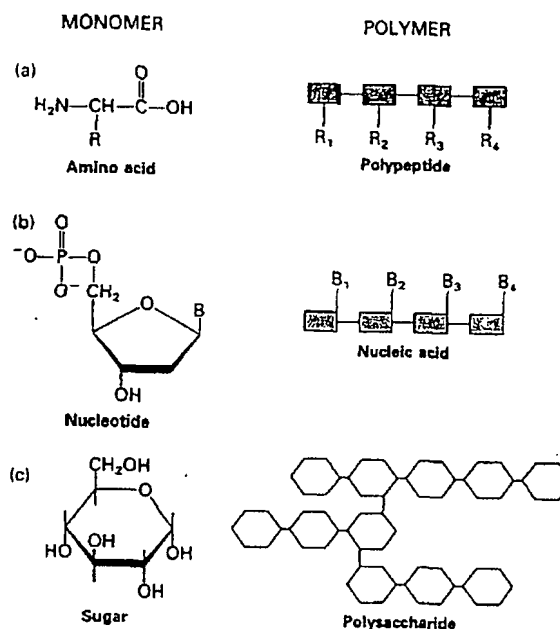
This chapter deals with the structures and some functions of biopolymers and small molecules; later chapters describe how the polymers are made and consider many of their other functions and interactions. ▲

## Proteins

Proteins are the working molecules of the cell. They catalyze an extraordinary range of chemical reactions, provide structural rigidity, control membrane permeability, regulate the concentrations of metabolites, recognize and noncovalently bind other biomolecules, cause motion, and control gene function. These incredibly diverse tasks are performed by molecules constructed from only 20 different amino acids.

### Amino Acids—the Building Blocks of Proteins—Differ Only in Their Side Chains

The monomers that make up proteins are called amino acids because, with one exception, each contains an *amino group* ( $-\text{NH}_2$ ) and an *acidic carboxyl group* ( $-\text{COOH}$ ). The exception, proline, has an *imino group* ( $-\text{NH}-$ ) instead of an amino group. At typical pH values in cells, the amino and carboxyl groups are ionized as  $-\text{NH}_3^+$  and  $-\text{COO}^-$ . All amino acids are constructed according to a basic design: a central carbon atom, called the  $\alpha$  carbon  $\text{C}_\alpha$  (because it is adjacent to the acidic carboxyl group), is bonded to an amino (or imino) group, to the carboxyl group, to a hydrogen atom, and to one variable group, called a *side chain* or *R group* (Figure 2-2). The side chains give the amino acids their individuality.



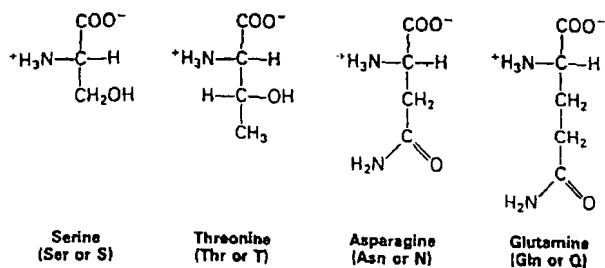
▲ **Figure 2-1** (a) Proteins, linear biopolymers called polypeptides, are formed from monomeric subunits termed amino acids. Each of the 20 different amino acids has a different R group, or side chain. Thus the polypeptide shown here, which is constructed of four amino acids, has  $20^4$ , or 160,000, possible structures. (b) Nucleic acids, also linear biopolymers, are formed from four monomers termed nucleotides, each of which has a different nitrogen-containing base structure (B). The nucleic acid shown here has  $4^4$ , or 256, possible structures. (c) Polysaccharides are built of monomeric saccharide (sugar) subunits. Because sugar residues can bind to one another at different positions, nonlinear branching polymers are often formed. The rings in (b) and (c) are depicted as Haworth projections (planar structures with a hint of perspective).

The amino acids represent the alphabet in which linear proteins are “written”; any student of biology must be familiar with the special properties of each letter of this alphabet. These letters can be classified into a few distinct categories.

The side chains of four of the amino acids are highly ionized and therefore charged at neutral pH. *Arginine* and *lysine* are positively charged; *aspartic acid* and *glutamic acid* are negatively charged and exist as aspartate and glutamate. The side chain of a fifth amino acid, *histidine*, is positively charged, but only weakly at neutral pH. In many cases, arginine may substitute for lysine, or aspartate for glutamate, with little effect on the structure or function of the protein.

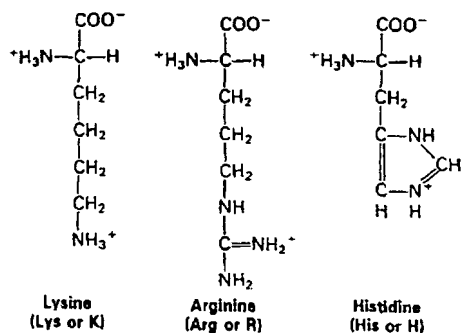
*Serine* and *threonine*, whose side chains have an  $-\text{OH}$  group, can interact strongly with water by forming hydrogen bonds. The side chains of *asparagine* and *gluta-*

## POLAR BUT UNCHARGED R GROUPS

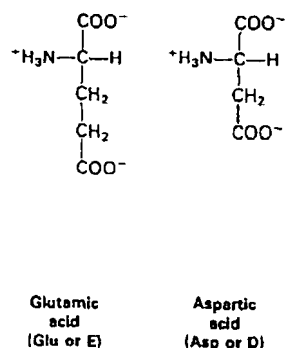


▼ **Figure 2-2** The structures of the 20 common amino acids. In each structure, a central carbon atom (the  $\alpha$  carbon) is bonded to an amino group (or to an imino group in proline), a carboxyl group, a hydrogen atom, and an R group. The R groups are in red.

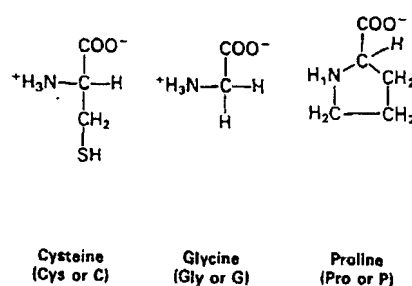
## POSITIVELY CHARGED R GROUPS



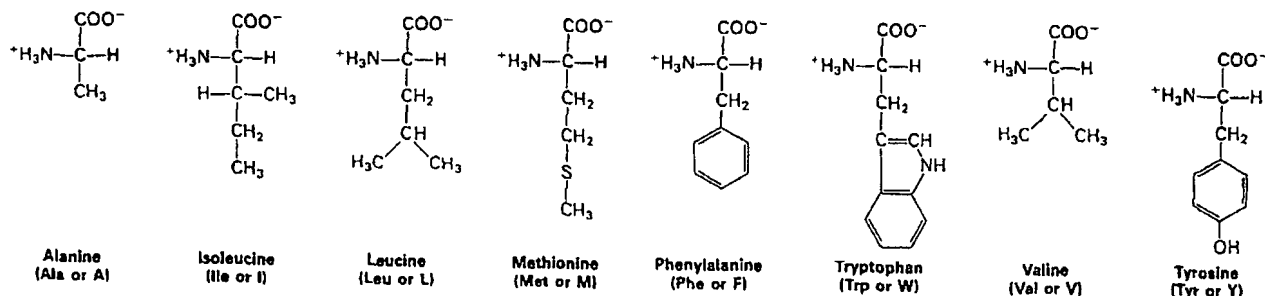
## NEGATIVELY CHARGED R GROUPS



## SPECIAL AMINO ACIDS



## HYDROPHOBIC R GROUPS



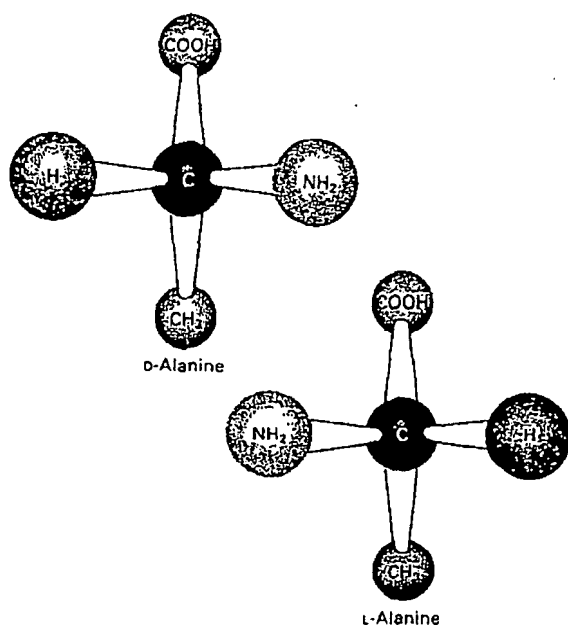
*mine* have polar amide groups with even more extensive hydrogen-bonding capacities. Together with the charged amino acids, these amino acids constitute the nine hydrophilic or polar amino acids.

The side chains of several other amino acids—*alanine*, *isoleucine*, *leucine*, *methionine*, *phenylalanine*, *tryptophan*, and *valine*—consist only of hydrocarbons, except for the sulfur atom in methionine and the nitrogen atom in tryptophan. These nonpolar amino acids are hydrophobic; their side chains are only slightly soluble in water. *Tyrosine* is also strongly hydrophobic because of its benzene ring, but its hydroxyl group allows it to interact with water, making its properties somewhat ambiguous.

*Cysteine* plays a special role in proteins because its  $-SH$  group allows it to dimerize through an  $-S-S-$  bond to a second cysteine, thus covalently linking regions of polypeptide to one another. When the  $-SH$  remains free, cysteine is quite hydrophobic.

Two other special amino acids are *glycine* and *proline*. Glycine has a hydrogen atom as its R group; thus it is the smallest amino acid and has no special hydrophobic or hydrophilic character. Proline, as an imino acid, is very rigid and creates a fixed kink in a polypeptide chain. It is quite hydrophobic.

The structure of all amino acids except glycine are asymmetrically arranged around the  $\alpha$  carbon, because it is bonded to four different atoms or groups of atoms

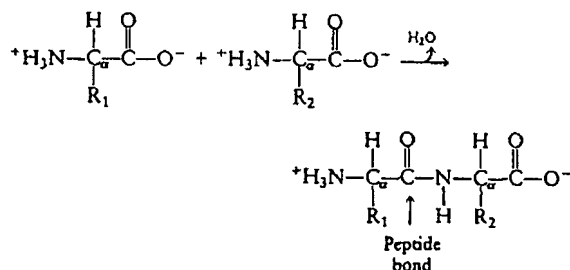


▲ Figure 2-3 Stereoisomers of the amino acid alanine. The  $\alpha$  carbon is black.

( $-\text{NH}_2$ ,  $-\text{COOH}$ ,  $-\text{H}$ , and  $-\text{R}$ ). Thus all amino acids except glycine can have one of two stereoisomeric forms. By convention, these mirror-image structures are called the D and the L forms of the amino acid (Figure 2-3). They cannot be interconverted without breaking a chemical bond. With rare exceptions, only the L forms of amino acids are found in proteins.

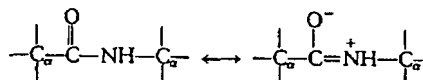
### Polypeptides Are Polymers Composed of Amino Acids Connected by Peptide Bonds

The *peptide bond*, the chemical bond that connects two amino acids in a polymer, is formed between the amino group of one amino acid and the carboxyl group of another. This reaction, called *condensation*, liberates a water molecule:



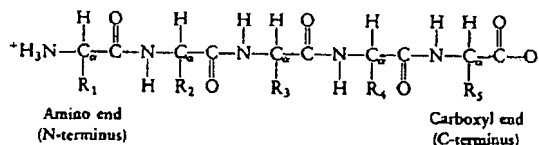
Because the carboxyl carbon and oxygen atoms are connected by a double bond, the peptide bond between car-

bon and nitrogen exhibits a partial double-bond character, as shown by the resonance structures



making it shorter than the typical C—N single bond. The six atoms of the peptide group (the two carbons of the adjacent amino acids and the carbon, oxygen, nitrogen, and hydrogen atoms of the bond) lie in the same plane (Figure 2-4a). However, adjacent peptide groups are not necessarily coplanar, due to rotation about the C— $\text{C}_\alpha$  and N— $\text{C}_\alpha$  bonds (Figure 2-4b).

A single linear array of amino acids connected by peptide bonds is called a *polypeptide*. If the polypeptide is short (fewer than 30 amino acids long), it may be called an *oligopeptide* or just a *peptide*. Polypeptides in living cells differ greatly in length; they generally contain between 40 and 1000 amino acids. Each polypeptide has a free amino group at one end (the N-terminus) and a free carboxyl group at the other (the C-terminus):

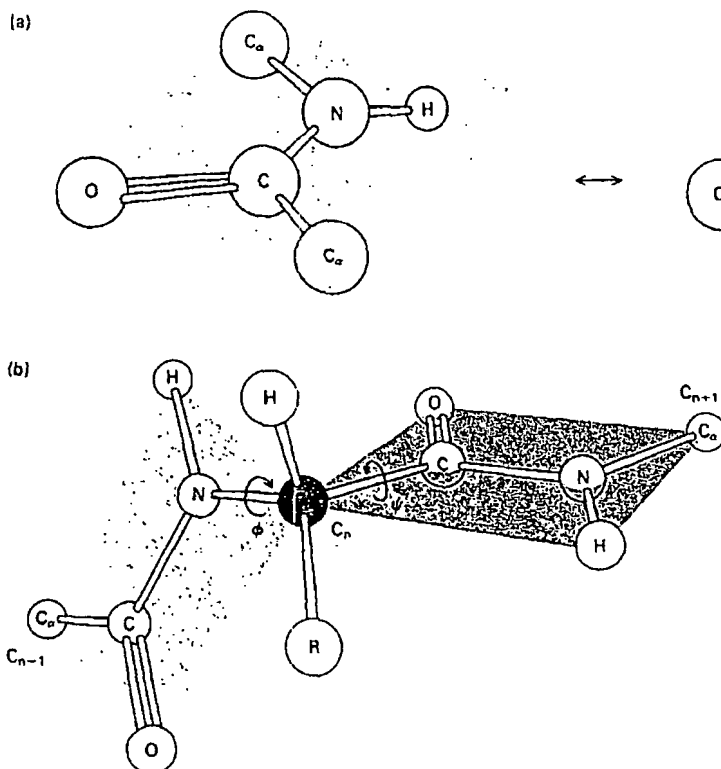


A protein is not merely a linear string of amino acids. The polypeptide folds up to form a specific three-dimensional structure that can be a long rod, as in the *fibrous proteins* that give tissues their rigidity, or a compact ball called a *globular protein*, as in many proteins that catalyze chemical reactions (enzymes), or a combination of balls and rods. The polypeptide can be modified further by the covalent or noncovalent attachment of additional small molecules.

A protein adopts a stable, folded conformation mainly through noncovalent (ionic, hydrogen, van der Waals, and hydrophobic) interactions. Its stability is also enhanced by the formation of covalent disulfide bonds between cysteines in different parts of the chain. Proteins may also consist of multiple polypeptide chains held together by noncovalent forces and, in some cases, by disulfide bonds. A well-characterized example is the hemoglobin molecule, which consists of four chains: two identical  $\alpha$  chains and two identical  $\beta$  chains (Figure 2-5).

### Three-dimensional Protein Structure Is Determined through X-ray Crystallography

The detailed three-dimensional structures of numerous proteins have been established by the painstaking efforts of many workers—notably, Max Perutz and John Kendrew, who perfected the x-ray crystallography of



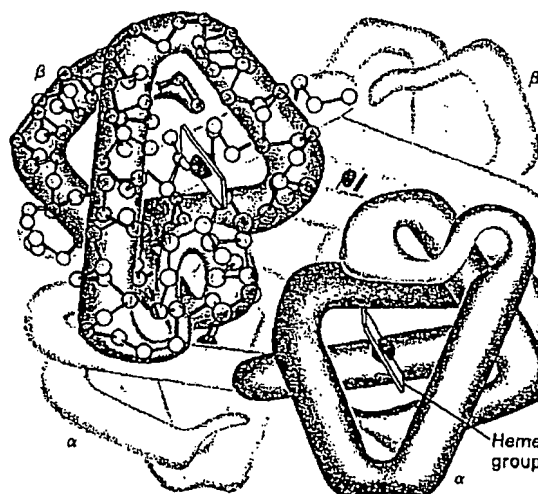
◀ **Figure 2-4** (a) Because the carbon-nitrogen peptide bond has a partial double-bond character, the peptide group is planar. (b) However, there is considerable flexibility in the geometry of polypeptides: rotation is possible about the two covalent single bonds that connect each  $\alpha$  carbon to the two adjacent planar peptide units. But some restrictions do apply to the values of  $\psi$  and  $\phi$ . For example, if the pictured adjacent peptide groups were coplanar, then certain oxygen and hydrogen atoms would be separated by less than their van der Waals radii and would repel one another.

proteins, in which beams of x-rays are passed through a crystal of protein. The wavelengths of x-rays are about 0.1–0.2 nanometers (nm)—short enough to resolve the atoms in the protein crystal. The three-dimensional structure of the protein can be deduced from the *diffraction pattern* of discrete spots that is produced when the scattered radiation is intercepted by photographic film. Such patterns are extremely complex; as many as 25,000 diffraction spots can be obtained from a small protein. Elaborate calculations and modifications of the protein (such as binding of heavy metal) must be made to interpret the diffraction pattern and to solve the structure of the protein.

Recently, three-dimensional structures of some small proteins have been determined by nuclear magnetic resonance (nmr) methods. An advantage of this approach is that it avoids the need to crystallize the protein. A disadvantage is that it is limited to relatively small proteins (up to about 20,000 molecular weight).

### The Structure of a Polypeptide Can Be Described at Four Levels

The structures adopted by polypeptides can be divided into four levels of organization. *Primary structure* refers to the linear arrangement of amino acid residues along a



▲ **Figure 2-5** The conformations assumed by the two  $\alpha$  and two  $\beta$  chains in a molecule of hemoglobin. Each chain forms several  $\alpha$  helices (see Figure 2-6). Only the backbones formed by the carbon and nitrogen atoms of the chains are shown here. A multitude of noncovalent interactions stabilize the conformations of the individual chains and the contacts between them. A heme group is bound to each chain. After R. E. Dickerson and I. Geis, 1969, *The Structure and Action of Proteins*, Benjamin-Cummings, p. 56. Copyright 1969 by Irving Geis.

polypeptide chain and to the locations of covalent bonds (mainly —S—S— bonds) between chains. *Secondary structure* pertains to the folding of parts of these chains into regular structures, such as  $\alpha$  helices and  $\beta$  pleated sheets. *Tertiary structure* includes the folding of regions between  $\alpha$  helices and  $\beta$  pleated sheets, as well as the combination of these secondary features into compact shapes (domains). *Quaternary structure* refers to the organization of several polypeptide chains into a single protein molecule, such as in hemoglobin.

### Two Regular Secondary Structures Are Particularly Important

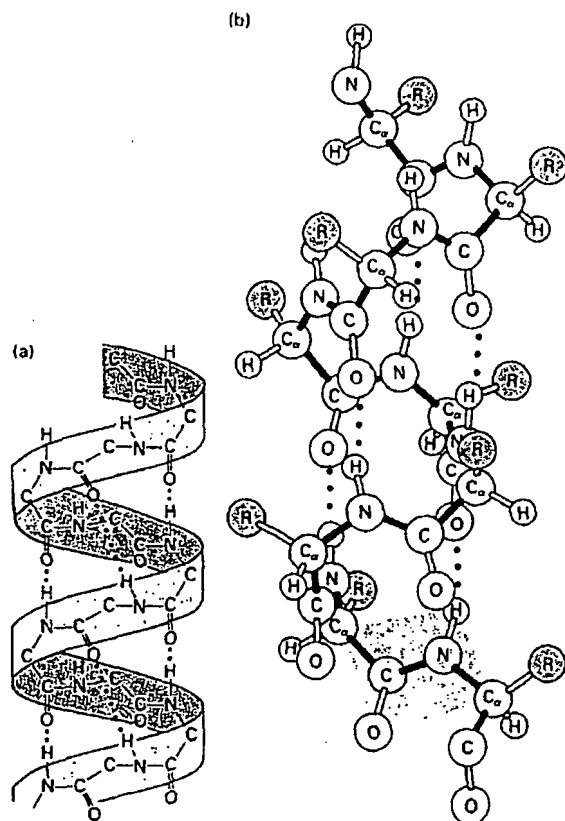
**The  $\alpha$  Helix** Although some regions of proteins are held in unique and irregular conformations, much protein structure involves repeated use of a limited number of regular configurations. One common structure, the  $\alpha$  helix, was first described by Linus Pauling and Robert B. Corey in 1951. Through careful model building, these scientists came to realize that polypeptide seg-

ments composed of certain amino acids tend to arrange themselves in regular helical conformations. In an  $\alpha$  helix, the carboxyl oxygen of each peptide bond is hydrogen-bonded to the hydrogen on the amino group of the fourth amino acid away (Figure 2-6), so that the helix has 3.6 amino acids per turn. Each amino acid residue represents an advance of about 1.5 Å along the axis of the helix. Every C=O and N—H group in the peptide bonds participates in a hydrogen bond, and the rigid planarity of the peptide bonds contributes to the rigid shape of the helix. In this inflexible, stable arrangement of amino acids, the side chains are positioned along the outside of a cylinder. The hydrogen-bonding potential of the peptide bonds is entirely satisfied internally, so that the polar or nonpolar quality of the cylindrical surface is determined entirely by the side chains. At least some of the amino acids in most proteins are organized into  $\alpha$  helices.

Certain amino acid sequences adopt the  $\alpha$ -helical conformation more readily than others. What determines this propensity is complicated, but some simple factors are evident. For instance, proline is rarely found in  $\alpha$ -helical regions because it cannot use its peptide nitrogen to make a hydrogen bond. Glycine also is an infrequent participant. Another inhibiting factor can be the tendency of multiple identically charged residues to repel each other.

The  $\alpha$  helix is a rodlike element of protein structure that serves many functions. A globular protein can be made up of short  $\alpha$ -helical rods connected by bends that allow the rods to interact with each other; hemoglobin, for instance, is 70 percent  $\alpha$  helical (see Figure 2-5). Alternatively, a single rod can span a long distance, as in the protein on the surface of the influenza virus (Figure 2-7a). Even in extended molecules, a, b, c the  $\alpha$  helix is usually found packed against other elements of protein, not as an isolated structure. Long fibers, such as the skin protein keratin or the muscle protein myosin (Figure 2-7b), can be formed by two or three  $\alpha$  helices that wrap gently around each other to form *coiled coils*. Small rods of  $\alpha$  helix interact with DNA in some DNA-binding proteins (Figure 2-7c). A helical rod bearing only hydrophobic side chains can span lipid membranes well because the hydrophilic peptide bonds are buried inside the helix.

Many  $\alpha$  helices are *amphipathic*: they expose hydrophilic side chains on one face and hydrophobic side chains on another face. Looking down the central axis of an  $\alpha$  helix (Figure 2-8a), the amino acid residues are arranged in a wheel; if the helix is amphipathic, most or all



◀ **Figure 2-6** Models of the  $\alpha$  helix. (a) This ribbonlike representation without R groups emphasizes the helical form. (b) This ball-and-stick representation emphasizes the role of the individual atoms and shows the R groups (green) that protrude from the helix body at regular intervals. Some of the planes of the  $C_\alpha$ —CO—NH groups are shaded orange. Part (b) after L. Stryer, 1988, *Biochemistry*, 3d ed., W. H. Freeman and Company, p. 26.

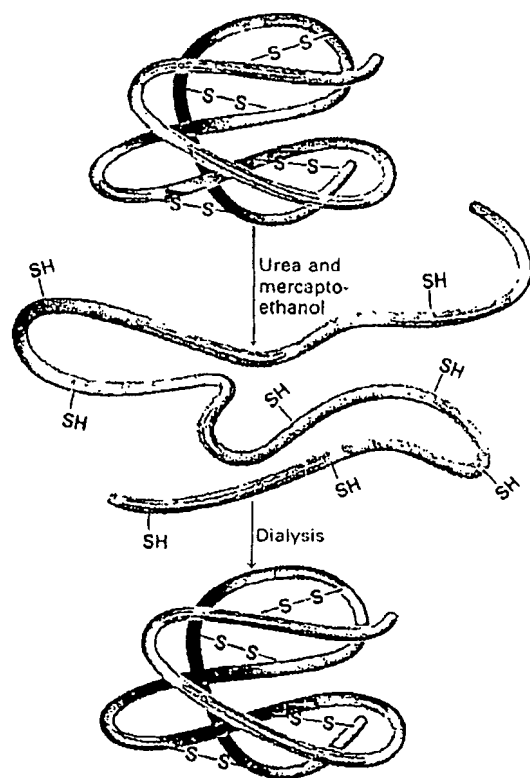


valently bound prosthetic group. For example, staphylococcal nuclease—a bacterial enzyme of 149 residues that degrades DNA and RNA—is totally denatured in acid but renatures to its native conformation within 0.1 s after the solution is neutralized. The three-dimensional architecture of this protein is solely a consequence of interactions among its amino acids and with its aqueous environment. In such cases, the genetic program of the cell must only define the primary structure of the protein—the amino acid sequence—and the tertiary structure is assured. With care, most proteins can be carried through a denaturation-renaturation cycle. Thus it is generally true that linear structure determines three-dimensional architecture.

The native form of some proteins is not the conformation with the lowest free energy and consequently cannot be completely restored on renaturation. This is particu-

larly true of multichain proteins. The two chains of insulin, for example, can be separated by a combination of reducing agents (to break the disulfide bridges) and concentrated solutions of such chemicals as urea (to disrupt hydrogen and hydrophobic bonds). When the insulin renatures in the presence of oxidizing agents that promote the formation of disulfide bridges, a number of stable multichain aggregates do form, but *native* insulin molecules make up only a minor proportion of them. In the others, the re-formed disulfide bridges connect inappropriate parts of the chain.

Insulin is formed by the partial proteolysis (breaking down) of proinsulin, its larger precursor (see Figure 2-13). Denatured proinsulin, as opposed to the denatured two-chain form of insulin, can renature to form the native structure of proinsulin with a high efficiency. Presumably, within the cell, either proinsulin or preproinsulin folds in such a way that the correct disulfide bridges form at the lowest free energy. The cell utilizes these intermediate stages to form insulin, whose stable conformation is not the one of lowest free energy.



▲ **Figure 2-15** Denaturation and renaturation of a protein. Most polypeptides can be completely unfolded by treatment with an 8 M urea solution containing mercaptoethanol ( $\text{HSCH}_2\text{CH}_2\text{OH}$ ). The urea breaks intramolecular hydrogen and hydrophobic bonds, and the mercaptoethanol reduces each disulfide bridge to two  $-\text{SH}$  groups. When these chemicals are removed by dialysis, the  $-\text{SH}$  groups on the unfolded chain oxidize spontaneously to re-form disulfide bridges, and the polypeptide chain simultaneously refolds into its native configuration.

## Enzymes

Protein catalysts called *enzymes* are mediators of the dynamic events of life; almost every chemical reaction in a cell is catalyzed by an enzyme. Like other catalysts, enzymes increase the rates of reactions that are already energetically favorable; more precisely, enzymes increase the rates of forward and reverse reactions by the same factor. The name of an enzyme usually indicates its function: the suffix *-ase* is commonly appended to the name of the type of molecule on which the enzyme acts. Thus proteases degrade proteins, phosphatases remove phosphate residues, and ribonuclease cleaves RNA molecules.

The chemicals that undergo a change in a reaction catalyzed by an enzyme are the *substrates* of that enzyme. Because little free energy may be liberated in either direction in reversible reactions, the distinction between chemicals that are substrates and those that are products is often arbitrary.

Most enzymes are found inside cells, but a number are secreted by cells and function in the blood, the digestive tract, or other extracellular spaces. In microbial species, some enzymes function outside the organism. The number of different types of chemical reactions in any one cell is very large: an animal cell, for example, normally contains 1000–4000 different types of enzymes, each of which catalyzes a single chemical reaction or set of closely related reactions. Certain enzymes are found in the majority of cells because they catalyze common cellular reactions—the synthesis of proteins, nucleic acids, and phospholipids and the conversion of glucose and oxygen into carbon dioxide and water, which produces most of the chemical energy used in animal cells. Other enzymes are

found only in a particular type of cell within an organism, such as a liver cell or a nerve cell, because they carry out some chemical reaction unique to that cell. Also, many mature cells, including erythrocytes (red blood cells) and epidermal (skin) cells, may no longer be capable of making proteins or nucleic acids yet these cells still contain specific sets of enzymes that they synthesized at an earlier stage of differentiation.

### Certain Amino Acids in Enzymes Bind Substrates: Others Catalyze Reactions on the Bound Substrates

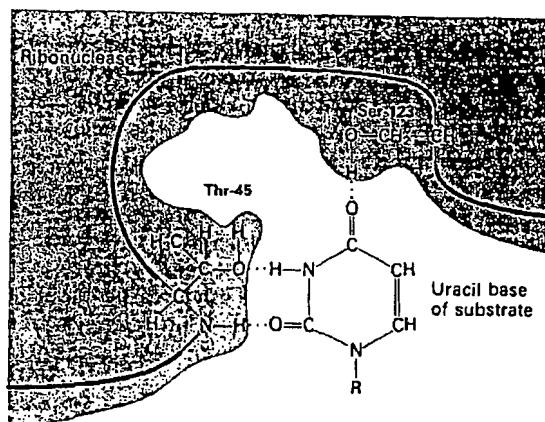
Two striking properties characterize all enzymes: their enormous *catalytic power* and their *specificity*. Quite often, the rate of an enzymatically catalyzed reaction is  $10^6$ – $10^{12}$  times that of an uncatalyzed reaction under otherwise similar conditions. The specificity of an enzyme is determined by the different rates at which it catalyzes closely similar chemical reactions or by its ability to distinguish between closely similar substrates.

Certain amino acid side chains of an enzyme are important in determining its specificity and its ability to accelerate the reaction rate. The properties of an enzyme are thus functions of its linear arrangement of amino acids and of the appropriate foldings of the peptide chain. Enzyme molecules have two important regions, or sites: one that recognizes and binds the substrate(s), and one that catalyzes the reaction once the substrate(s) have been bound. The amino acids in each of these key regions do not need to be adjacent in the linear polypeptide; they are brought into proximity in the folded molecule. In some enzymes, the catalytic site is part of the substrate-binding site. These two regions are called, collectively, the *active site*.

The binding of a substrate to an enzyme usually involves the formation of multiple noncovalent ionic, hydrogen, and hydrophobic bonds and van der Waals interactions (Figure 2-16). The array of chemical groups in the active site of the enzyme is precisely arranged so that the specific substrate can be more tightly bound than any other molecule (with the exception of some enzyme inhibitors) and the reaction can occur readily. In catalysis, covalent bonds between the enzyme and the substrate may be formed (and then broken) to reduce the activation energy for the reaction.

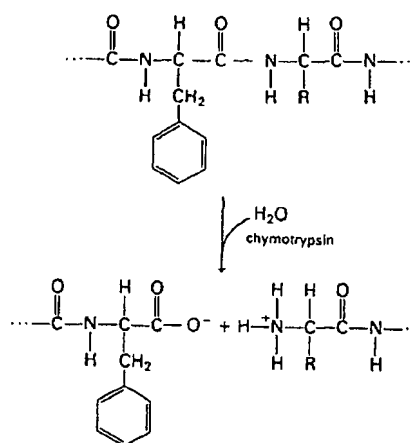
### Trypsin and Chymotrypsin Are Well-characterized Proteolytic Enzymes

The proteolytic (protein-digesting) enzymes trypsin and chymotrypsin are synthesized in the pancreas and secreted into the small intestine as inactive precursors, or *zymogens*, called trypsinogen and chymotrypsinogen, respectively. These zymogens are not activated until they reach the small intestine where they hydrolyze peptide

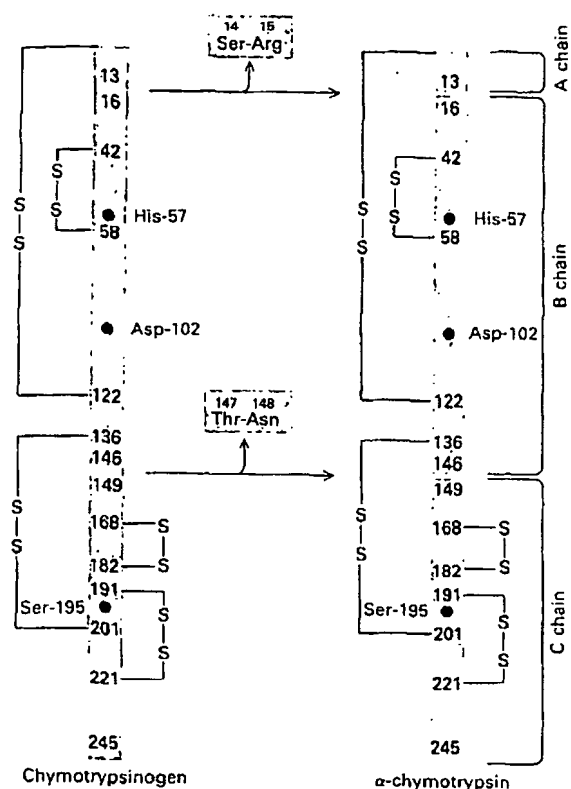


▲ **Figure 2-16** The specific binding of a substrate to an enzyme involves the formation of multiple noncovalent bonds. Here, two amino acid residues of the enzyme ribonuclease bind uracil, part of its substrate, by three hydrogen bonds. Substrates without the two C=O groups and one N—H group in the appropriate positions would be unable to bind or would bind less tightly. Other regions of the enzyme, not depicted here, bind other parts of the RNA substrate by hydrogen bonds and van der Waals interactions.

bonds of ingested proteins—a step in their digestion to single amino acids (Figure 2-17). The delay in activation serves an important regulatory purpose: it prevents the enzyme from digesting the pancreatic tissue in which it was made. Two irreversible proteolytic cleavages activate chymotrypsin. One cleavage removes serine 14 (the serine at position 14) and arginine 15 from chymotrypsinogen; the other removes threonine 147 and asparagine 148



▲ **Figure 2-17** The hydrolysis of a peptide bond by chymotrypsin.



▲ **Figure 2-18** A linear representation of the conversion of chymotrypsinogen into chymotrypsin by the excision of two dipeptides. The positions of the disulfide bridges are indicated. In the folded molecule, histidine 57, aspartate 102, and serine 195 are located in the active site.

(Figure 2-18). Removal of these two dipeptides activates the protease function of the enzyme.

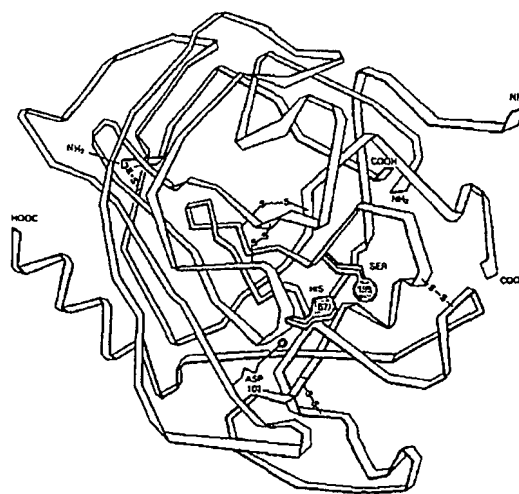
The hydrolysis of peptide bonds is energetically favorable ( $\Delta G^\circ = -2$  kcal/mol). Nonetheless, the activation energy for an *uncatalyzed* peptide-bond hydrolysis—say, in a neutral aqueous solution of a protein at room temperature—is so high that there is little or no hydrolysis even after several months. Biochemists can chemically hydrolyze proteins into their constituent amino acids by treating them with a 6 M solution of hydrochloric acid in an evacuated tube at 100°C for 24 h. Yet at 37°C and neutral pH, a molecule of trypsin or chymotrypsin can catalyze the hydrolysis of up to 100 peptide bonds per second. The power of enzymatically mediated catalysis is well-illustrated here: the addition of sufficient enzyme can do in seconds what otherwise would require harsh conditions and long times.

Chymotrypsin does not hydrolyze all peptide bonds; rather, it is selective for the peptide bond at the carboxyl ends of amino acids such as phenylalanine, tyrosine, and

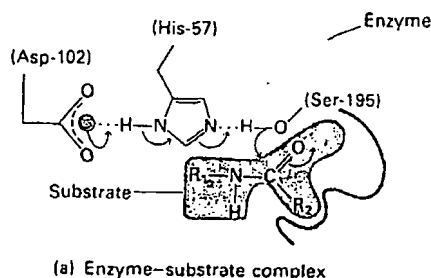
tryptophan, which have large hydrophobic side chains. Trypsin, by contrast, is specific for the peptide bond on the C-terminal side of lysine and arginine residues.

**Specific Amino Acid Side Chains of Chymotrypsin Aid in Substrate Binding** The reaction mechanism of chymotrypsin was deduced, in part, from the three-dimensional structure obtained by x-ray crystallography (Figure 2-19). The enzyme contains three polypeptides—the A, B, and C chains, which have 13, 131, and 97 amino acids, respectively. These chains are interconnected by disulfide bridges (see Figures 2-18 and 2-19). The molecule has two key structural features: the active site and the *hydrophobic cleft* (a crevice bordered by the side chains of several hydrophobic amino acid residues), which serves as the binding site for specific amino acid residues on the substrate. The conformation of this pocket allows the residues lining it to participate in hydrophobic interactions with the large hydrophobic side chains of phenylalanine, tyrosine, or tryptophan. Neither charged side chains nor small hydrophobic residues on the substrate can make the noncovalent bonds necessary to fit into the cleft.

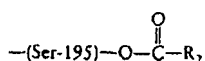
The hydrophobic residues of most globular proteins are buried in the interior; when such proteins are in their native states, the peptide bonds linking the hydrophobic residues are not accessible to hydrolysis by chymotrypsin. Normally, stomach acids (pH 1) denature ingested proteins so that proteases in that organ can partly degrade them before their exposure to further digestion by chymotrypsin at neutral pH in the intestine.



▲ **Figure 2-19** A three-dimensional model of  $\alpha$ -chymotrypsin determined from x-ray analysis. The N- and C-termini of the A, B, and C chains are indicated, as are the —S—S— bridges and the three amino acid residues of the active site (red). After B. W. Matthews et al., 1967, *Nature* 214:652.

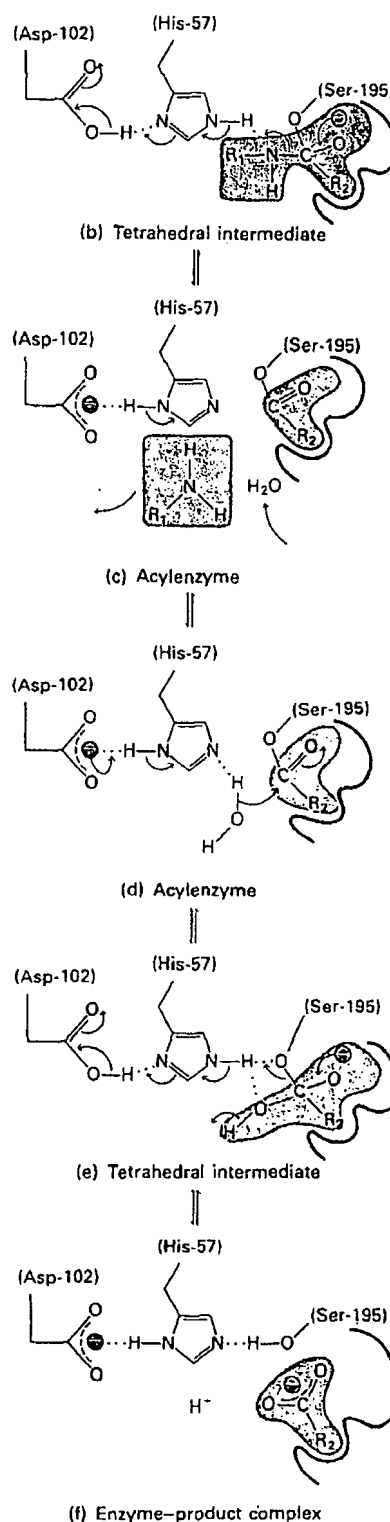


▲ **Figure 2-20** The mechanism of hydrolysis of a peptide bond by  $\alpha$ -chymotrypsin. Red curved arrows represent the movement of electrons. (a) The substrate is bound to the enzyme so that the bond to be hydrolyzed is positioned near serine 195. The negative charge (blue) surrounding the oxygens in aspartate 102 induces a charge relay system, which is initiated when the oxygen atoms on Asp-102 attract a proton from the nitrogen atom on His-57. When the negative charge reaches the second nitrogen in His-57, the nitrogen removes the proton from the hydroxyl group on Ser-195. The resulting  $O^-$  attacks the carbon of the bound substrate to form (b) a tetrahedral intermediate, so called because the carbon atom of interest temporarily has four single bonds. The hydrogen bound to the second nitrogen in His-57 is then added to the nitrogen of the substrate. As a result, the C-N bond of the substrate breaks, leaving (c)  $R_1NH_2$  and the acyl-enzyme intermediate

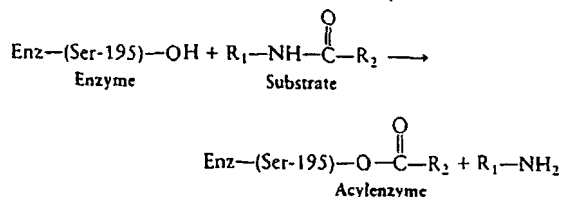


The  $R_1NH_2$  is discharged from the enzyme and replaced by water. In the resulting structure (d), a similar charge relay system is induced, and His-57 removes a proton from the hydrogen-bonded  $H_2O$ . The  $OH^-$  thus generated attacks the carboxyl carbon of the acyl-enzyme to form (e) another tetrahedral intermediate. The bond between the tetrahedral carbon and the oxygen of Ser-195 is hydrolyzed to yield (f)  $R_2COO^-$  bound noncovalently to the free enzyme, from which it is released. After R. M. Stroud, et al., 1975, in *Proteases and Biological Control*, E. Reich et al., eds. Cold Spring Harbor Laboratory, p. 25.

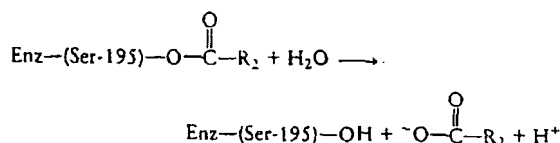
**Other Amino Acid Side Chains of Chymotrypsin Have Roles in Catalyzing the Hydrolysis of the Bound Substrate** The catalytic activity of chymotrypsin depends on three amino acid residues: histidine 57, aspartate 102, and serine 195. These amino acids are distant from one another in the primary structure of the protein (see Figure 2-18), but the chains are folded in such a way in the active enzyme molecule that the three side chains are close together, in the correct position for catalyzing the hydrolysis of a peptide bond in a protein bound to the enzyme (see Figure 2-19). When chymotrypsinogen is proteolytically activated, the polypeptide conformation is altered to bring these three residues into correct alignment.



The hydrolysis reaction proceeds in two main steps. First, the peptide bond is broken and the carboxyl group is transferred to the hydroxyl residue of serine 195:



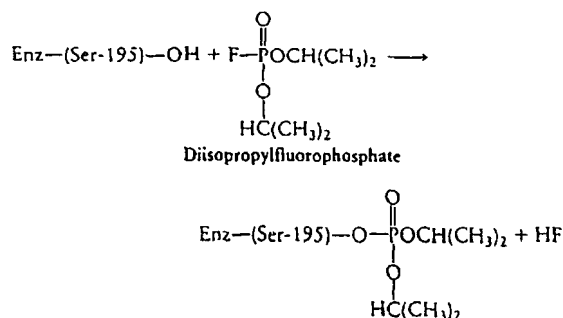
Second, this *acylenzyme* intermediate is hydrolyzed:



Note that the second step restores the enzyme to its original state.

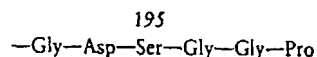
Aspartate 102 and histidine 57 facilitate the acylation reaction by removing the proton from serine 195 and adding it to the nitrogen of the departing amino group (Figure 2-20). In a similar manner, aspartate 102 and histidine 57 facilitate the hydrolysis of the acylenzyme. These enzymatically catalyzed steps—transfer of a proton from the enzyme to the substrate, formation of a covalent acylserine intermediate, and hydrolysis of the acylenzyme—all drastically reduce the overall activation energy of the proteolysis reaction.

The hydroxyl group on serine 195 is unusually reactive. The concept of an “active” serine residue at the active site predated the determination of the crystal structure of chymotrypsin. It was already known, for example, that the compound diisopropylfluorophosphate is a potent inhibitor of chymotrypsin; it reacts only with the hydroxyl on serine 195 to form a stable covalent compound that irreversibly inactivates the enzyme:



**Trypsin and Chymotrypsin Have Different Substrate-binding Sites** A comparison of trypsin and chymotrypsin will emphasize the nature of the specificity of enzymatically catalyzed reactions. About 40 percent of the amino acids in these two molecules are the same; in

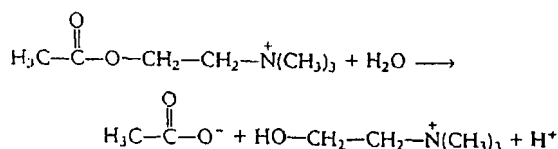
particular, the amino acid sequences in the vicinity of the key serine residue are identical:



The three-dimensional structures and catalytic mechanisms of these two enzymes are also quite similar, indicating that they evolved from a common polypeptide. The major difference between trypsin and chymotrypsin is found in the side chains of the amino acids that line the substrate-binding site. The negatively charged amino acids in this area of the trypsin molecule facilitate the binding of only positively charged (lysine or arginine) residues, instead of hydrophobic ones.

### Other Hydrolytic Enzymes Contain Active Serine

Other, mostly unrelated, hydrolytic enzymes also contain an active serine residue that is essential for catalysis. For example, acetylcholinesterase catalyzes the hydrolysis of the neurotransmitter acetylcholine to acetate and choline:



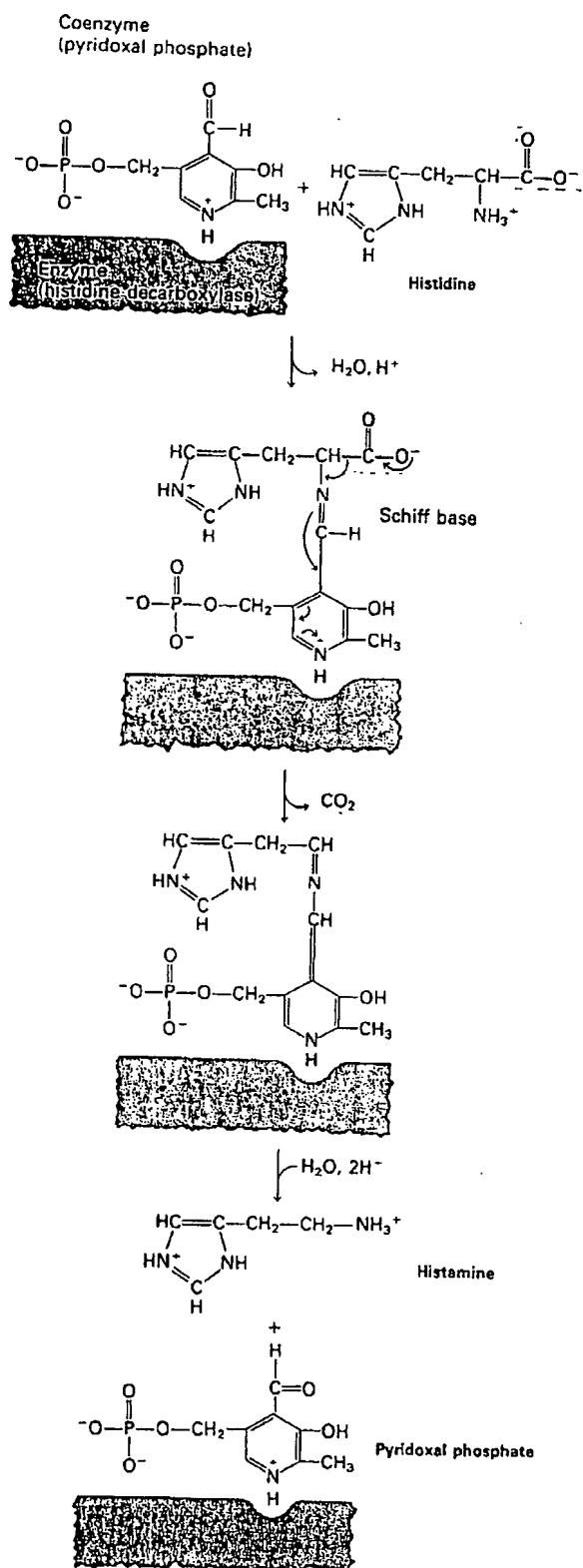
Diisopropylfluorophosphate is a potent, irreversible inhibitor of acetylcholinesterase as well as of chymotrypsin. The compound is lethal to animals because it blocks nerve transmission by causing a buildup of the transmitter substance. (The action of this transmitter is discussed in Chapter 20.)

### Coenzymes Are Essential for Certain Enzymatically Catalyzed Reactions

Many enzymes contain a *coenzyme*—a tightly bound small molecule or prosthetic group essential to enzymatic activity. Vitamins required in trace amounts in the diet are often converted to coenzymes. Coenzyme A, for instance, is derived from the vitamin pantothenic acid; the coenzyme pyridoxal phosphate is derived from vitamin B<sub>6</sub>. To cite just one example of how coenzymes function, we consider pyridoxal phosphate. The aldehyde group



can form a covalent complex called a *Schiff base* with an  $-\text{NH}_2$  group of an amino acid, which facilitates or lowers the activation energy for the breaking of bonds to the carbon of the amino acid. Figure 2-21 shows how pyridoxal phosphate catalyzes the decarboxylation of histidine to form histamine—a potent dilator of small blood vessels. Histamine is released by certain cells in the course of allergic hypersensitivity.



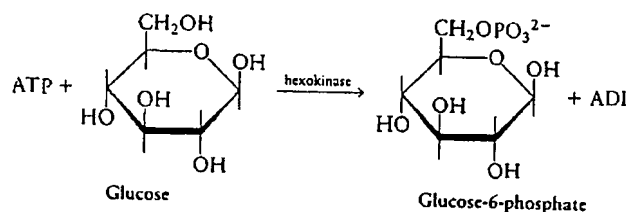
◀ **Figure 2-21** Pyridoxal phosphate, a coenzyme, participates in many reactions involving amino acids. When it is bound to histidine decarboxylase, as in this example, it forms a Schiff base with the  $\alpha$  amino group of histidine. The positive charge on the nitrogen of pyridoxal phosphate then attracts the electrons from the carboxylate group of the histidine, via a charge relay system. This weakens the bond between the  $\alpha$  carbon of the histidine and the carboxylate group, causing the release of  $\text{CO}_2$ . Finally, histamine, the reaction product, is hydrolyzed from the pyridoxal complex.

### Substrate Binding May Induce a Conformational Change in the Enzyme

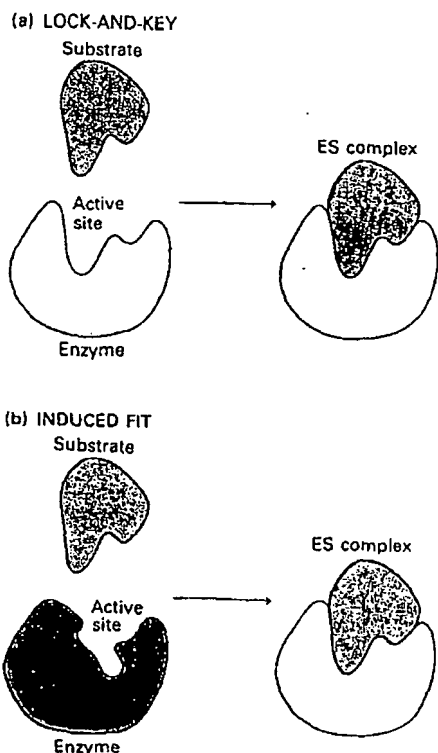
When a substrate binds to an enzyme, molecules of complementary charge or shape, or both, may simply fit together into a complex stabilized by a variety of noncovalent bonds. Such an interaction resembles the fitting of a key into a lock and is said to occur by a *lock-and-key* mechanism (Figure 2-22a).

In some enzymes, the binding of the substrate induces a conformational change in the enzyme that causes the catalytic residues to become positioned correctly. Molecules that attach to the substrate-binding site, or *recognition site*, of the enzyme but that do not induce a conformational change are not substrates of that enzyme. Thus an enzyme differentiates between a substrate and a nonsubstrate in two ways: Does the potential substrate bind to the enzyme? If so, does it induce the correct conformational change? When both criteria are met, the enzyme-substrate complex is said to demonstrate *induced fit* (Figure 2-22b).

An important example of induced fit is provided by the enzyme hexokinase, which catalyzes the transfer of a phosphate residue from ATP to a specific carbon atom of glucose:



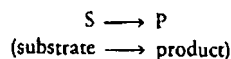
This is the first step in the degradation of glucose by cells. X-ray crystallography has shown that hexokinase consists of two domains. The binding of glucose induces a major conformational change that brings these domains closer together and creates a functional catalytic site (Figure 2-23). Only glucose and closely related molecules can induce this conformational change, ensuring that the enzyme is used to phosphorylate only the correct substrates. Molecules such as glycerol, ribose, and even water may bind to the enzyme at the recognition site but cannot induce the requisite conformational change, so they are not substrates for the enzyme.



▲ **Figure 2-22** Two mechanisms for the interaction of an enzyme and a substrate. (a) In the lock-and-key mechanism, the substrate fits directly into the binding site of the enzyme. (b) If binding occurs by induced fit, the substrate induces a conformational change in the enzyme that appropriately positions the substrate for catalysis.

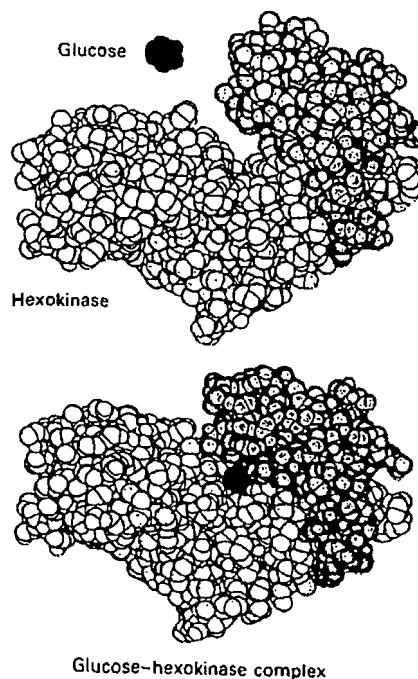
### The Catalytic Activity of an Enzyme Can Be Characterized by a Few Numbers

Enzymatic specificity is usually quantified by discrimination ratios: a good substrate may be cleaved 10,000 times as fast as a poor substrate. The catalytic power of an enzyme on a given substrate involves two numbers:  $K_m$ , which measures the affinity of the enzyme for its substrate, and  $V_{max}$ , which measures the maximal velocity of enzymatic catalysis. Equations for  $K_m$  and  $V_{max}$  are most easily derived by considering the simple reaction



in which the rate of product formation depends on  $[S]$ , the concentration of the substrate, and on  $[E]$ , the concentration of the catalytic enzyme. For an enzyme with a single catalytic site, Figure 2-24(a) shows how  $d[P]/dt$ , the rate of product production, depends on  $[S]$  when  $[E]$  is kept constant.

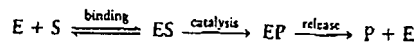
At low concentrations of  $S$ , the reaction rate is propor-



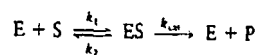
▲ **Figure 2-23** The conformation of hexokinase changes markedly when it binds the substrate glucose: the two domains of the enzyme come closer together to surround the substrate. Molecules such as the five-carbon sugar ribose can also bind to hexokinase by forming specific hydrogen bonds with groups in the substrate-binding pocket of the enzyme, but only glucose can form all of the bonds that cause the enzyme to change its conformation. Courtesy of Dr. Thomas A. Steitz.

tional to  $[S]$ ; as  $[S]$  is increased the rate does not increase indefinitely in proportion to  $[S]$  but eventually reaches  $V_{max}$ , at which it becomes independent of  $[S]$ .  $V_{max}$  is proportional to  $[E]$  and to a catalytic constant  $k_{cat}$  that is an intrinsic property of the individual enzyme; halving  $[E]$  reduces the rate at all values of  $[S]$  by one-half.

When interpreting curves such as those in Figure 2-24, bear in mind that all enzymatically catalyzed reactions include at least three steps: (1) the binding of the substrate ( $S$ ) to the enzyme ( $E$ ) to form an enzyme-substrate complex ( $ES$ ); (2) the conversion of  $ES$  to the enzyme-product complex ( $EP$ ); and (3) the release of the product ( $P$ ) from  $EP$ , to yield free  $P$ :



In the simplest case, the release of  $P$  is so rapid that we can write



The reaction rate  $d[P]/dt$  is proportional to the concentration of ES and to the catalytic constant  $k_{cat}$  for the given enzyme:

$$\frac{d[P]}{dt} = k_{cat} [ES] \quad (1)$$

To calculate [ES], we assume the reaction is in a steady state, so that  $k_1 [E] [S]$ , the formation rate of [ES], is equal to the rate of its consumption, either by dissociation of uncatalyzed substrate at a rate of  $k_2 [ES]$  or by catalysis at a rate of  $k_{cat} [ES]$ :

$$k_1 [E] [S] = (k_2 + k_{cat}) [ES] \quad (2)$$

If

$$[E]_{tot} = [E] + [ES] \quad (3)$$

(where  $[E]_{tot}$  is the sum of the free and the complexed enzyme, or the total amount of enzyme), then we can combine equations (2) and (3) to obtain

$$\begin{aligned} [E]_{tot} &= [E] + [ES] = \frac{(k_2 + k_{cat})}{k_1 [S]} [ES] + [ES] \\ &= [ES] \left[ 1 + \left( \frac{k_2 + k_{cat}}{k_1} \right) \left( \frac{1}{[S]} \right) \right] \end{aligned}$$

If we define  $K_m$ , called the *Michaelis constant*, as

$$\frac{k_2 + k_{cat}}{k_1} \quad (4)$$

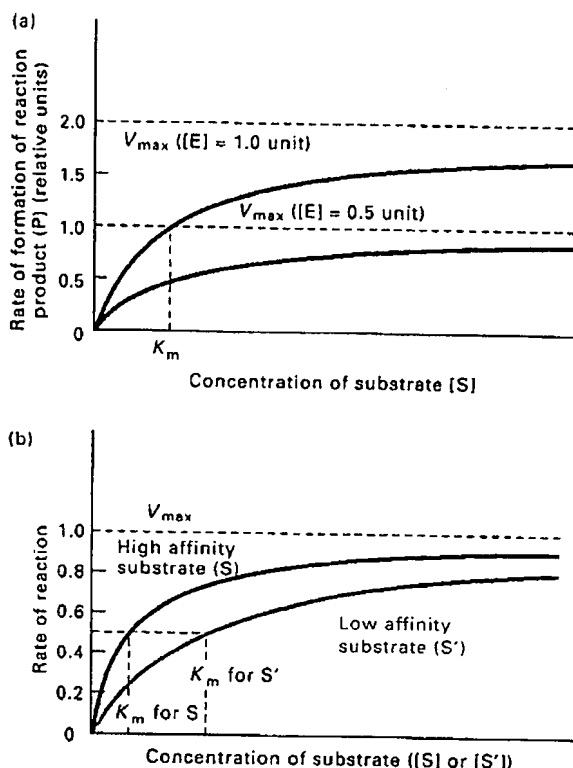
then

$$[ES] = \frac{[E]_{tot}}{1 + K_m/[S]}$$

Thus

$$\begin{aligned} \frac{d[P]}{dt} &= k_{cat} [ES] = k_{cat} [E]_{tot} \frac{1}{1 + K_m/[S]} \\ &= k_{cat} [E]_{tot} \frac{[S]}{[S] + K_m} \end{aligned} \quad (5)$$

This equation fits the curves shown in Figure 2-24a.  $V_{max}$ , which is equal to  $k_{cat} [E]_{tot}$ , is the maximal rate of product formation if all recognition sites on the enzyme are filled with substrate.  $K_m$  is equivalent to the substrate concentration at which the reaction rate is half-maximal. (If  $[S] = K_m$ , then from equation (5) we calculate the rate of product formation to be  $\frac{1}{2} k_{cat} [E]_{tot} = \frac{1}{2} V_{max}$ .) For most enzymes, the slowest step is the catalysis of [ES] to [E] + [P]. In these cases,  $k_{cat}$  is much less than  $k_2$ , so that  $K_m = (k_2 + k_{cat})/k_1 \approx k_2/k_1$  is equal to the equilibrium constant for binding S to E. Thus the parameter  $K_m$  describes the affinity of an enzyme for its substrate. The smaller the value of  $K_m$ , the more avidly the enzyme can bind the substrate from a dilute solution (Figure 2-24b) and the lower the value of [S] needed to reach half-maximal velocity. The concentrations of the various



▲ **Figure 2-24** (a) The rate of a hypothetical enzymatically catalyzed reaction  $S \rightarrow P$  for two different concentrations of enzyme [E] as a function of the concentration of substrate [S]. The substrate concentration that yields a half-maximal reaction rate is denoted by  $K_m$ . Doubling the amount of enzyme causes a proportional increase in the rate of the reaction, so that the maximal velocity  $V_{max}$  is doubled. The  $K_m$ , however, is unaltered. (b) The rates of reactions catalyzed by an enzyme with a substrate S, for which the enzyme has a high affinity, and with a substrate S', for which the enzyme has a low affinity. The  $V_{max}$  value is the same for S and S', but  $K_m$  is higher for S'.

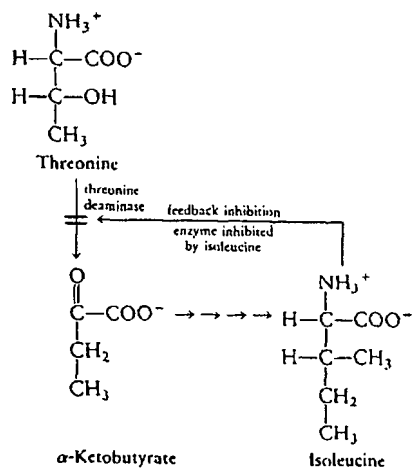
small molecules in a cell vary widely, as do the  $K_m$  values for the different enzymes that act on them. Generally, the intracellular concentration of a substrate is approximately the same as or greater than the  $K_m$  value of the enzyme to which it binds.

### The Actions of Most Enzymes Are Regulated

Many reactions in cells do not occur at a constant rate. Instead, the catalytic activity of the enzymes is *regulated* so that the amount of reaction product is just sufficient to meet the needs of the cell.



**An Enzyme Can Be Feedback Inhibited in a Reaction Pathway** Consider a series of reactions leading to the synthesis of the amino acid isoleucine, which is primarily used by cells as a monomer in the synthesis of proteins. The amount of isoleucine needed depends on the rate of protein synthesis in the cell. The first step in the synthesis of isoleucine is the elimination of an amino group, which converts the amino acid threonine to the compound  $\alpha$ -ketobutyrate. Threonine deaminase—the enzyme that catalyzes this reaction—plays a key role in regulating the level of isoleucine. In addition to its substrate-binding sites for threonine, threonine deaminase contains a binding site for isoleucine. When isoleucine is bound there, the enzyme molecule undergoes a conformational change, so that it cannot function as efficiently. Thus isoleucine acts as an *inhibitor* of the reaction for the conversion of threonine. If the isoleucine concentration in the cell is high, the binding of isoleucine to the enzyme temporarily reduces the rate of isoleucine synthesis:



This is an example of *feedback inhibition*, whereby an enzyme that catalyzes one of a series of reactions is inhibited by the ultimate product of the pathway.

In isoleucine synthesis, as in most cases of feedback inhibition, the final product in the reaction pathway inhibits the enzyme that catalyzes the first step that does not also lead to other products. The suppression of enzyme function is not permanent. If the concentration of free isoleucine is lowered, bound isoleucine dissociates from the enzyme, which then reverts to its active conformation. The binding of the inhibitor isoleucine to the enzyme and its subsequent release can be described by the equilibrium-binding constant  $K_i$ , which is similar to the constant  $K_m$  used for substrate binding:

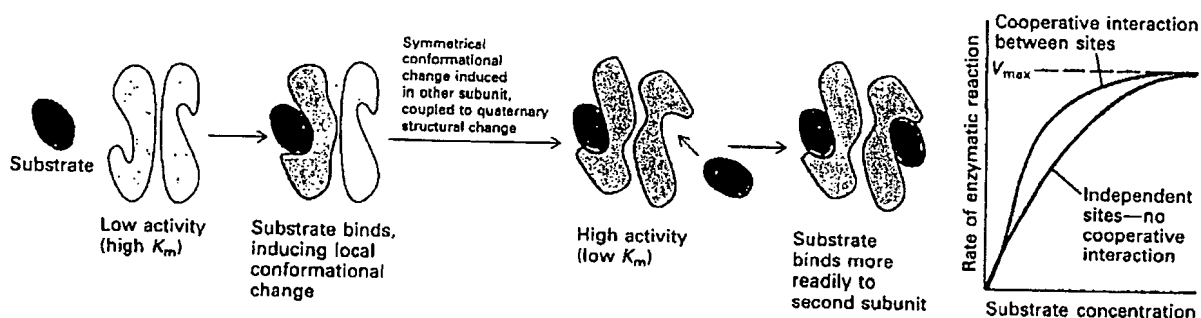
$$[\text{E} \cdot \text{Ile}]_{\text{inactive}} \xrightleftharpoons{K_i} [\text{Ile}] + [\text{E}]_{\text{active}}$$

$$K_i = \frac{[\text{Ile}][\text{E}]_{\text{active}}}{[\text{E} \cdot \text{Ile}]_{\text{inactive}}}$$

**Many Enzymes Have Multiple Binding Sites for Regulatory Molecules** Some enzymes have binding sites for small molecules that affect their catalytic activity; a stimulator molecule is called an *activator*. Enzymes may even have multiple sites for recognizing more than one activator or inhibitor. In a sense, enzymes are like microcomputers; they can detect concentrations of a variety of molecules and use that information to vary their own activities. Molecules that bind to enzymes and increase or decrease their activities are called *effectors*. Effectors can modify enzymatic activity because enzymes can assume both active and inactive conformations: activators are positive effectors; inhibitors are negative effectors. Effectors bind at *regulatory sites*, or *allosteric sites* (from the Greek for "another shape"), a term used to emphasize that the regulatory site is an element of the enzyme distinct from the catalytic site and to differentiate this form of regulation from competition between substrates and inhibitors at the catalytic site.

**Multimeric Organization Permits Cooperative Interactions among Subunits** Many enzymes and some other proteins are multimeric—that is, they contain several copies, or subunits, of one or more distinct polypeptide chains. Some multimeric enzymes contain identical subunits, each of which has a catalytic site and possibly an effector site. In other enzymes, regulatory sites and catalytic sites are located on different subunits, each with a particular structure. On binding an activator, inhibitor, or substrate, a subunit undergoes a conformational change, usually small, that triggers a change in quaternary structure. This quaternary rearrangement favors a similar conformational change in the other subunits, thereby increasing their affinity for the type of ligand initially bound (Figure 2-25). When several subunits interact cooperatively, a given increase or decrease in substrate or effector concentration causes a larger change in the rate of an enzymatic reaction than would occur if the subunits acted independently. Because of such *cooperative interactions*, a small change in the concentration of an effector or substrate can lead to large changes in catalytic activity.

Cooperative interactions among the four subunits in hemoglobin demonstrate clearly the advantages of multimeric organization. The binding of an  $\text{O}_2$  molecule to any one of the four chains (each hemoglobin chain binds one  $\text{O}_2$ ) induces a local conformational change in that subunit. This change can in turn induce a large change in quaternary structure. The quaternary change involves a rearrangement of the positions of the two  $\alpha$  and two  $\beta$  chains in the tetramer. The local conformational changes that accompany  $\text{O}_2$  binding can then occur more readily in the remaining subunits, increasing their affinity for oxygen. The binding of a second  $\text{O}_2$  makes the quaternary structural change even more likely. The cooperative

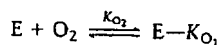


▲ **Figure 2-25** A cooperative interaction between active sites (two identical subunits of a hypothetical enzyme). The binding of a substrate to one subunit of a multimeric enzyme induces a conformational change in the adjacent subunit,

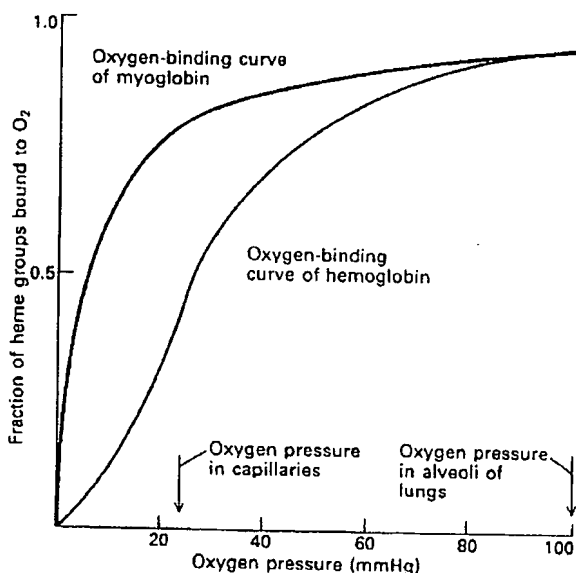
which lowers the  $K_m$  for the binding of the substrate there. Thus a small change in the substrate concentration can cause a much larger increase in the reaction rate than would occur if there were no cooperative interactions between active sites.

interaction between the chains causes the molecule to take up or lose four  $O_2$  molecules over a much narrower range of oxygen pressures than it would otherwise. As a result, hemoglobin is almost completely oxygenated at the oxygen pressure in the lungs and largely deoxygenated at the oxygen pressure in the tissue capillaries (Figure 2-26).

The contrast between hemoglobin and myoglobin is revealing. Myoglobin is a single-chain oxygen-binding protein found in muscle. The oxygen-binding curve of myoglobin has the characteristics of a simple equilibrium reaction:



Myoglobin has a greater binding affinity for  $O_2$  (a lower  $K_{O_2}$ ) than hemoglobin at all oxygen pressures. Thus, at



the oxygen pressure in capillaries,  $O_2$  moves from hemoglobin into the muscle cells, where it binds to myoglobin, ensuring the efficient transfer of  $O_2$  from blood to tissues.

The quaternary-structure rearrangements associated with multimeric organization also provide a way for the effects of activator or inhibitor binding at an allosteric site to be transmitted to a distant catalytic site without large changes in the secondary or tertiary structure of an enzyme, which would be incompatible with the principle that a particular primary structure must adopt a unique folded conformation. Thus, for example, small conformational changes in a domain in response to binding of an effector molecule would produce a quaternary-structure change, which amplifies the conformational signal and allows it to be transmitted robustly to other parts of the enzyme, where it would induce a small conformational change affecting enzymatic activity. Membrane-embedded receptor proteins that must transmit a conformational signal from one side of a membrane to the other are also likely to be multimeric; they transmit the signal by quaternary-structure rearrangement or by an effector-induced shift in the monomer-multimer equilibrium.

◀ **Figure 2-26** The binding of oxygen to hemoglobin depends on cooperative interactions between the four chains. The graph shows the fraction of heme groups in hemoglobin and in myoglobin bound to  $O_2$  as a function of the oxygen pressure. Note that the binding activity of hemoglobin increases sharply over a narrow range of oxygen pressures (20–40 mmHg). Hemoglobin is saturated with  $O_2$  in the lungs, but it releases much of its bound  $O_2$  at the low oxygen pressure in the tissue capillaries. At any oxygen pressure, myoglobin has a higher affinity for  $O_2$  than hemoglobin does. As myoglobin is a principal muscle protein, this property allows oxygen to be transferred from blood to muscle.

**Enzymes Are Regulated in Many Ways** The activities of enzymes are extensively regulated so that the numerous enzymes in a cell work together harmoniously. All metabolic pathways are closely controlled at all times. Synthetic reactions occur when the products of these reactions are needed; degradative reactions occur when molecules must be broken down. Kinetic controls affecting the activities of key enzymes determine which pathways are going to be used and the rates at which they will function.

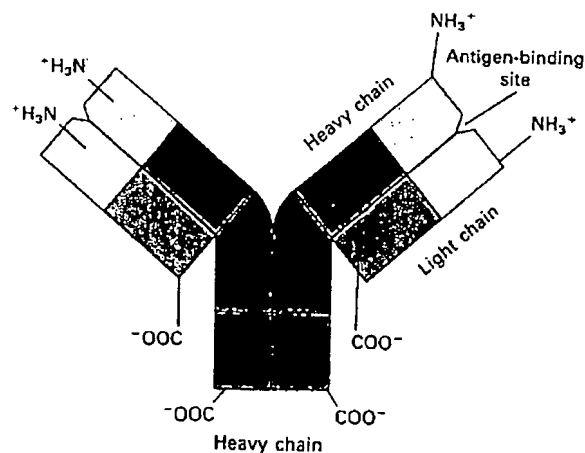
Regulation of cellular processes involves more than simply turning enzymes on and off, however. Some regulation is accomplished through *compartmentation*. Many enzymes are localized in specific compartments of the cell, such as the mitochondria or lysosomes, thereby restricting the substrates, effectors, and other enzymes with which an enzyme can interact. In addition, compartmentation permits reactions that might otherwise compete with one another in the same solution to occur simultaneously in different parts of a cell. Cellular processes are also regulated through the control of the rates of enzyme synthesis and destruction.

## Antibodies

Enzymes are not the only proteins that bind tightly and specifically to smaller compounds. The insulin receptor on the surface of a liver cell, for example, can bind to insulin so tightly that the receptors on a cell are half-saturated when the insulin concentration is only  $10^{-9}$  M. This protein does not bind to most other compounds present in blood; it mediates the specific actions of insulin on liver cells. A molecule other than an enzyme substrate that can bind specifically to a macromolecule is often called a *ligand* of that macromolecule.

The capacity of proteins to distinguish among different molecules is developed even more highly in blood proteins called *antibodies*, or *immunoglobulins*, than in enzymes. Animals produce antibodies in response to the invasion of an infectious agent, such as a bacterium or a virus. Antibodies will be discussed at length in Chapter 25. We introduce them here because they will appear as critical reagents in the discussions of many intervening chapters.

The recognition site of an antibody can bind tightly to very specific sites—generally on proteins or carbohydrates—on the surface of the infectious agent. Experimentally, animals produce antibodies in response to the injection of almost any foreign polymer; such antibodies bind specifically and tightly to the invading substance but, like enzymes, do not bind to dissimilar molecules. The antibody acts as a signal for the elimination of infectious agents. When it binds to a bacterium, virus, or virus-infected cell, certain white blood cells (leucocytes) recognize the invading body as foreign and respond by



▲ **Figure 2-27** The structure of an antibody molecule illustrated in an immunoglobulin (IgG) made of four polypeptide chains: two identical heavy chains (blue) and two identical light chains (orange). Each antigen-binding site is formed by the N-terminal segments of a heavy and a light chain. The N-termini are highly variable in sequence, giving rise to the wide range of antibody specificity.

destroying it. The specificity of antibodies is exquisite: they can distinguish between proteins that differ by only a single amino acid and between the cells of different individual members of the same species.

All vertebrates can produce a large variety of antibodies, including ones that bind to chemically synthesized molecules. Exposure to an antibody-producing agent, called an *antigen*, causes an organism to make a large quantity of different antibody proteins, each of which may bind to a slightly different region of the antigen. For a given antigen, these constellations of antibodies may differ from one member of a species to another.

Antibodies are formed from two types of polypeptides: heavy chains, each of which is folded into four domains, and light chains, each of which is folded into two domains (Figure 2-27). The N-terminal domains of both heavy and light chains are highly variable in sequence, giving rise to the specific binding characteristics of antibodies.

## Antibodies Can Distinguish among Closely Similar Molecules

The sequence of bovine insulin is identical to that of human insulin, except at three amino acids. Yet when bovine insulin is injected into people, some individuals respond by synthesizing antibodies that specifically recognize the specific amino acids in the bovine molecule, even though human beings generally do not produce anti-

# MICROBIOLOGY

## An Introduction

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*About the cover:* A technician is isolating plasmids, which are tiny circles of DNA found in bacteria. The plasmids are dissolved in a dye solution that fluoresces pink under ultraviolet light. Genetic engineering using plasmids is revolutionizing the biological sciences and industry (see pages 226-229 and 704-707).

Figure acknowledgments begin on page 749.

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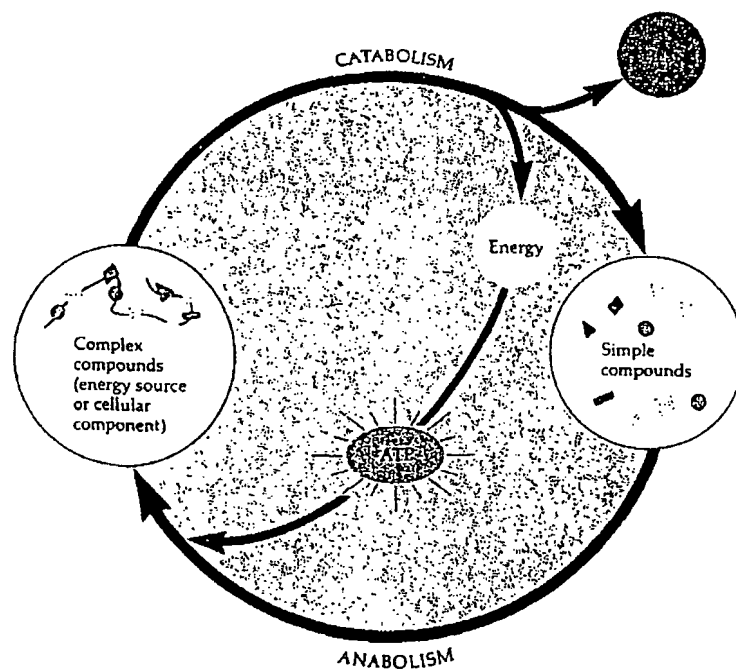
tial energy and therefore serve as energy carriers to drive energy-requiring reactions. The most common energy carrier in all biological systems is adenosine triphosphate (ATP); its structure can be reviewed in Figure 2-20. The role of ATP in the relationship between catabolic and anabolic processes is shown in Figure 5-1.

A little later in the chapter, we will examine some representative chemical reactions that deal with energy production (catabolic reactions) and energy utilization (anabolic reactions) in microorganisms. We will then look at how these various reactions are integrated within the cell. But first let us consider the principal properties of a group of proteins involved in almost all biologically important chemical reactions. These proteins, the enzymes, were described briefly in Chapter 2.

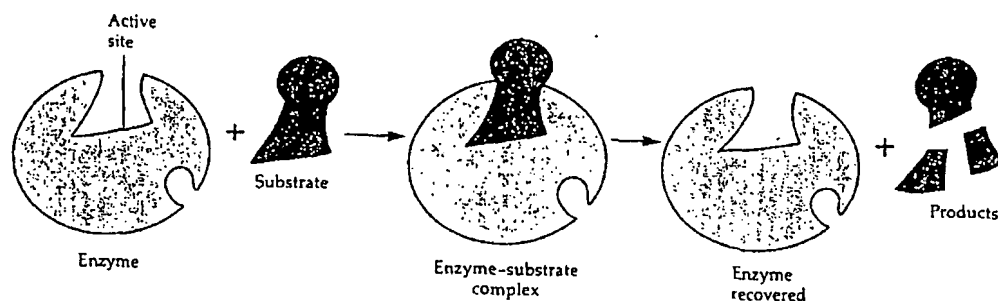
Although it is beyond the scope of this text to name and discuss the actions of individual enzymes, you should be aware of the central role of enzymes in metabolic reactions. It is important to understand that a cell's metabolic pathways are determined by its enzymes, which are, in turn, determined by its genetic makeup.

## ENZYMES

Many organic chemicals are so stable that they could remain unchanged in a cell for years. To activate these chemicals, living cells produce **enzymes**, proteins that act as catalysts in chemical reactions of importance to the cell. A *catalyst* is a substance that speeds up a reaction without being



**Figure 5-1** Relationship between anabolism and catabolism and the role of ATP. When simple compounds are combined to form complex compounds (anabolism), ATP provides the energy for synthesis. When large compounds are split apart (catabolism), heat energy is given off and some energy is trapped in ATP molecules.



**Figure 5-2** Mechanism of enzyme action. The surface of the substrate comes into contact with the active site on the surface of the enzyme to form an enzyme-substrate complex. The substrate is then transformed into products and the enzyme is recovered.

changed by it. Generally large globular proteins, enzymes range in molecular weight from about 10,000 to somewhere in the millions. Of the thousand or more known enzymes, each has a three-dimensional characteristic shape with a specific surface configuration due to its primary, secondary, and tertiary structures (see Figure 2-18).

### Mechanism of Enzyme Action

As mentioned in Chapter 2, catalysts lower the *activation energy* required for a chemical reaction. Although scientists do not completely understand how an enzyme does this, the sequence of events is believed to be as follows (Figure 5-2):

1. The surface of the *substrate*—that is, the molecule or molecules that are reactants in the chemical reaction to be catalyzed—contacts a specific region on the surface of the enzyme molecule, called the *active site*.
2. A temporary intermediate compound called an *enzyme-substrate complex* forms.
3. The substrate molecule is transformed (by rearrangement of existing atoms, a breakdown of the substrate molecule, or the combining of several substrate molecules).
4. The transformed substrate molecules, the products of the reaction, move away from the surface of the enzyme molecule.

5. The recovered enzyme, now freed, reacts with other substrate molecules.

Enzyme reaction is characterized by its extreme *specificity* for a particular substrate. For example, a specific enzyme may be capable of hydrolyzing a peptide bond only between two specific amino acids. And other enzymes are capable of hydrolyzing starch, but not cellulose; even though both starch and cellulose are polysaccharides composed of glucose subunits, the orientations of the subunits in the two polysaccharides differ. Enzyme specificity results from the three-dimensional shape of the active site, which fits the substrate somewhat like a lock with its key. In most instances, the substrate is much smaller than the enzyme, and relatively few of the enzyme's amino acids make up the active site.

A given compound can be a substrate for a number of different enzymes that catalyze different reactions. The fate of a given reactant (substrate) depends on the specific enzyme that reacts upon it. For example, glucose-6-phosphate, an important molecule in cell metabolism, may be acted upon by at least four different enzymes, each of which will give a different product.

Enzymes are exceedingly efficient. Under optimum conditions, they can catalyze reactions at rates that are  $10^6$  to  $10^{10}$  times (up to 10 billion times) more rapid than those of comparable reactions without enzymes. The *turnover number* (number of substrate molecules metabolized per enzyme mol-

Volume I

*Todd • Sanford • Davidsohn*

CLINICAL  
DIAGNOSIS *and*  
MANAGEMENT  
by  
LABORATORY  
METHODS

*Sixteenth Edition*

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destruction of the red cells with higher concentrations of the abnormal hemoglobin or selective removal of the abnormal hemoglobin from the cell.

In *heterozygous alpha hemoglobinopathies*, the abnormality in the alpha chain will affect all three hemoglobin types. Therefore, six different hemoglobin types are found—the three normal hemoglobins and the three abnormal forms. Examples are Hb D<sup>Baltimore</sup>, Hb Ann Arbor, and Hb M<sup>Boston</sup>.

Combinations of abnormalities exist. *Double heterozygotes for two beta chain abnormalities* produce two different abnormal beta chains; therefore, there are two abnormal hemoglobins and no hemoglobin A. An example of this is Hb S-C disease. Double heterozygotes for beta and delta chain abnormalities are rare but have provided important information. The latter will have four major hemoglobin types on electrophoresis:  $\alpha_2\beta_2^A\delta_2^A$ ;  $\alpha_2\beta_2^A\delta_2^S$ ;  $\alpha_2\beta_2^S\delta_2^A$ ; and  $\alpha_2\beta_2^S\delta_2^S$ .

*Double heterozygotes for beta hemoglobinopathy and beta thalassemia* are well known. Here, the quantity of abnormal hemoglobin exceeds the normal hemoglobin, in contrast to the heterozygous beta hemoglobinopathies, in which the reverse is true. Examples are Hb S thalassemias and Hb E thalassemia.

#### Beta hemoglobinopathies

Hemoglobins S, C, D, and E are believed to be polymorphisms because their frequency is greater than can be explained by mutation alone (Lehmann, 1977). They occur in homozygous as well as heterozygous form and involve the beta chain.

**Sickle Cell Disease.** Homozygous Hb S disease is a serious chronic hemolytic anemia, first manifest in early childhood and often fatal before the age of 30 years. With modern medical care, however, many patients live longer. Hemoglobin S is found almost exclusively in the black population; 0.1 to 0.2 per cent of the blacks born in the United States have sickle cell anemia (Schneider, 1976).

In hemoglobin S the glutamic acid in the sixth position on the beta chain is replaced by valine. This substitution is on the surface of the molecule and changes its charge and, hence, its electrophoretic mobility. Hemoglobin S is freely soluble when fully oxygenated; when oxygen is removed from Hb S, polymerization of the abnormal hemoglobin occurs, forming tactoids (fluid crystals) which are

rigid and deform the cell into the shape which gave the cell its name (Fig. 29-7). In homozygous Hb S disease, sickling occurs at physiologic oxygen tensions and the rigidity of the red cells is responsible for the hemolysis as well as for most of the complications. The rigid cells are more vulnerable to trauma and are readily trapped by the reticuloendothelial system, especially the spleen, accounting for the hemolysis. As a result of the hemolysis, severe continued marrow hyperplasia during childhood produces bone changes: expansion of the marrow space, thinning of the cortex, and radial striations seen in the skull on x-ray. Leg ulcers are common.

**COMPLICATIONS.** In early childhood, bilateral painful swelling of the dorsa of the hands or feet occurs as a result of sickling and capillary stasis; this is known as the *hand-foot syndrome* or sickle cell dactylitis. It lasts about two weeks, is accompanied by changes of periostitis as observed by x-ray, and does not occur after the age of four.

The spleen is central to three complications: *A sequestration crisis* refers to sudden pooling of blood and rapid enlargement of the spleen, resulting in hypovolemic shock. This may occur in early childhood when splenomegaly is present. *Functional asplenia* (Pearson, 1969) consists of inadequate antibody responses under some conditions and an impaired ability of the reticuloendothelial system to clear bacteria and particulate material from the blood, probably due to reticuloendothelial blockade. This may partly explain the increased risk of infection in children with the disease. Salmonella and pneumococcal infections are unusually prevalent in children with sickle cell anemia. *Autosplenectomy* is the result of vaso-occlusive episodes, resulting in progressive infarction, fibrosis, and contraction of the spleen. Though splenomegaly is present in childhood, a small fibrotic remnant is the rule in the adult.

From early childhood, patients cannot produce a concentrated urine, apparently as a result of anoxic damage to the vasa recta in the medullae of the kidneys. Hematuria as a result of papillary necrosis is common.

*Vaso-occlusive crises* are debilitating episodes of abdominal and bone or joint pain, accompanied by fever, which are probably due to plugging of small blood vessels by masses of sickled cells. Bone necrosis occurs and may be a focus for salmonella osteomyelitis. Aseptic necrosis of the femoral head is occasionally a complication. The various complications as a

This order is not binding precedent of the Board.

Paper 103

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BEFORE THE BOARD OF PATENT APPEALS  
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Human Genome Sciences, Inc.  
Junior Party  
(Patent 6,872,568;  
Inventors: Jian Ni, Reiner L. Gentz,  
Guo-Liang Yu, Craig A. Rosen),

v.

Genentech, Inc.,  
Senior Party  
(Application 10/423,448;  
Inventors: Camella W. Adams, Avi J. Ashkenazi,  
Anan Chuntharapai, Kyung Jin Kim).

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Patent Interference No. 105,361 (RES)

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Richard E. Schafer, *Administrative Patent Judge*.

**ORDER- Bd. R. 104(a)**

**ADE- 39**  
USSN 10/052,798

1 During a conference call held March 13, 2007, each party sought  
2 permission to file a motion seeking to change the subject matter of the  
3 interference. HGS sought authorization to file a motion to add two pending  
4 applications, one assigned to each party, to the interference. Genentech  
5 sought authorization to file a motion to declare an additional interference  
6 between the applications.

7        Neither application was in condition for allowance, although it was  
8        represented that the applications would be in condition for allowance soon.

9 No motions were authorized.

10           The parties were required to file a notice when their respective  
11 applications were in condition for allowance. At that time, the declaration of  
12 an additional interference will be considered.

13           The examiners assigned to the applications are aware of the potential  
14   interference.

/Richard E. Schafer/ ) BOARD OF PATENT  
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5 al., J. Exp. Med., 176:1191-1195 (1992); Shopes, J. Immunol., 148:2918-2922 (1992). Homodimeric antibodies may also be prepared using heterobifunctional cross-linkers as described in Wolff et al., Cancer Research, 53:2560-2565 (1993). Ghetie et al., Proc. Natl. Acad. Sci., 94:7509-7514 (1997), further describe preparation of IgG-IgG homodimers and disclose that such homodimers can enhance apoptotic activity as compared to the monomers. Alternatively, the antibodies can be engineered to have dual Fc regions [see, Stevenson et al., Anti-Cancer Drug Design, 3:219-230 (1989)].

15 D. Therapeutic and Non-therapeutic Uses for Apo-2 Antibodies

20 The Apo-2 antibodies of the invention have therapeutic utility. Agonistic Apo-2 antibodies, for instance, may be employed to activate or stimulate apoptosis in cancer cells. The Apo-2 antibodies of the invention may also be useful in enhancing immune-mediated cell death in cells expressing Apo-2, for instance, through complement fixation or ADCC. Alternatively, antagonistic antibodies may be used to block excessive apoptosis (for instance in neurodegenerative disease) or to block potential autoimmune/inflammatory effects of Apo-2 resulting from NF-KB activation.

25 Apo-2 antibodies may further be used in diagnostic assays for Apo-2, e.g., detecting its expression in specific cells, tissues, or serum. Various diagnostic assay techniques known in the art may be used, such as competitive binding assays, direct or indirect sandwich assays and immunoprecipitation assays conducted in either heterogeneous or homogeneous phases [Zola, Monoclonal Antibodies: A Manual of Techniques, CRC Press, Inc. (1987) pp. 147-158]. The antibodies used in the diagnostic assays can be labeled with a detectable moiety. The detectable moiety should be capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety may be a radioisotope, such as <sup>3</sup>H, <sup>14</sup>C, <sup>32</sup>P, <sup>35</sup>S, or <sup>125</sup>I, a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin, or an enzyme, such as alkaline phosphatase, beta-galactosidase or horseradish peroxidase. Any method known in the art for conjugating the antibody to the detectable moiety may be employed, including those methods described by Hunter et al., Nature, 144:945 (1962); David et al., Biochemistry, 13:1014 (1974); Pain et

5 al., J. Immunol. Meth., 40:219 (1981); and Nygren, J. Histochem. and Cytochem., 30:407 (1982).

10 Apo-2 antibodies also are useful for the affinity purification of Apo-2 from recombinant cell culture or natural sources. In this process, the antibodies against Apo-2 are immobilized on a suitable support, such a Sephadex resin or filter paper, using methods well known in the art. The immobilized antibody then is contacted with a sample containing the Apo-2 to be purified, and thereafter the support is washed with a suitable solvent that will remove substantially all the material in the sample except the Apo-2, which is bound to the  
15 immobilized antibody. Finally, the support is washed with another suitable solvent that will release the Apo-2 from the antibody.

#### E. Kits Containing Apo-2 or Apo-2 Antibodies

20 In a further embodiment of the invention, there are provided articles of manufacture and kits containing Apo-2 or Apo-2 antibodies which can be used, for instance, for the therapeutic or non-therapeutic applications described above. The article of manufacture comprises a container with a label. Suitable containers include, for example, bottles, vials, and test tubes. The containers may be formed from  
25 a variety of materials such as glass or plastic. The container holds a composition which includes an active agent that is effective for therapeutic or non-therapeutic applications, such as described above. The active agent in the composition is Apo-2 or an Apo-2 antibody. The label on the container indicates that the composition is used for a  
30 specific therapy or non-therapeutic application, and may also indicate directions for either *in vivo* or *in vitro* use, such as those described above.

35 The kit of the invention will typically comprise the container described above and one or more other containers comprising materials desirable from a commercial and user standpoint, including buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

\*\*\*\*\*

40 The following examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

All patent and literature references cited in the present

5 specification are hereby incorporated by reference in their entirety.

#### EXAMPLES

10 All restriction enzymes referred to in the examples were purchased from New England Biolabs and used according to manufacturer's instructions. All other commercially available reagents referred to in the examples were used according to manufacturer's instructions unless otherwise indicated. The source of those cells identified in the following examples, and throughout the specification, by ATCC accession numbers is the American Type Culture Collection, Rockville, Maryland.

15

#### EXAMPLE 1

##### Isolation of cDNA clones Encoding Human Apo-2

20 Expressed sequence tag (EST) DNA databases (LIFESEQ™, Incyte Pharmaceuticals, Palo Alto, CA) were searched and an EST was identified which showed homology to the death domain of the Apo-3 receptor [Marsters et al., Curr. Biol., 6:750 (1996)]. Human pancreas and kidney lgt10 bacteriophage cDNA libraries (both purchased from Clontech) were ligated into pRK5 vectors as follows. Reagents were added together and incubated at 16°C for 16 hours: 5X T4 ligase buffer (3 ml); pRK5, Xho1, 25 Not1 digested vector, 0.5 mg, 1 ml); cDNA (5 ml) and distilled water (6 ml). Subsequently, additional distilled water (70 ml) and 10 mg/ml tRNA (0.1 ml) were added and the entire reaction was extracted through phenol:chloroform:isoamyl alcohol (25:24:1). The aqueous phase was removed, collected and diluted into 5M NaCl (10 ml) and absolute ethanol 30 (-20°C, 250 ml). This was then centrifuged for 20 minutes at 14,000 x g, decanted, and the pellet resuspended into 70% ethanol (0.5 ml) and centrifuged again for 2 minutes at 14,000 x g. The DNA pellet was then dried in a speedvac and eluted into distilled water (3 ml) for use in the subsequent procedure.

35 The ligated cDNA/pRK5 vector DNA prepared previously was chilled on ice to which was added electrocompetent DH10B bacteria (Life Tech., 20 ml). The bacteria vector mixture was then electroporated as per the manufacturers recommendation. Subsequently SOC media (1 ml) was added and the mixture was incubated at 37°C for 30 minutes. The 40 transformants were then plated onto 20 standard 150 mm LB plates containing ampicillin and incubated for 16 hours (37°C) to allow the

5 colonies to grow. Positive colonies were then scraped off and the DNA isolated from the bacterial pellet using standard CsCl-gradient protocols.

10 An enriched 5'-cDNA library was then constructed to obtain a bias of cDNA fragments which preferentially represents the 5' ends of cDNA's contained within the library. 10 mg of the pooled isolated full-length library plasmid DNA (41 ml) was combined with Not 1 restriction buffer (New England Biolabs, 5 ml) and Not 1 (New England Biolabs, 4 ml) and incubated at 37°C for one hour. The reaction was extracted through phenol:chloroform:isoamyl alcohol (25:24:1, 50 ml), the aqueous phase removed, collected and resuspended into 5M NaCl (5 ml) and absolute ethanol (-20°C, 150 ml). This was then centrifuged for 20 minutes at 14,000 x g, decanted, resuspended into 70% ethanol (0.5 ml) and centrifuged again for 2 minutes at 14,000 x g. The supernatant was then removed, the pellet dried in a speedvac and resuspended in distilled water (10 ml).

20 The following reagents were brought together and incubated at 37°C for 2 hours: distilled water (3 ml); linearized DNA library (1 mg, 1 ml); Ribonucleotide mix (Invitrogen, 10 ml); transcription buffer (Invitrogen, 2 ml) and Sp6 enzyme mix. The reaction was then extracted through phenol:chloroform:isoamyl alcohol (25:24:1, 50 ml) and the aqueous phase was removed, collected and resuspended into 5M NaCl (5 ml) and absolute ethanol (-20°C, 150 ml) and centrifuged for 20 minutes at 14,000 x g. The pellet was then decanted and resuspended in 70% ethanol (0.5 ml), centrifuged again for 2 minutes at 14,000 x g, decanted, dried in a speedvac and resuspended into distilled water (10 ml).

30 The following reagents were added together and incubated at 16°C for 16 hours: 5X T4 ligase buffer (Life Tech., 3 ml); pRK5 Cla-Sal digested vector, 0.5 mg, 1 ml); cDNA (5 ml); distilled water (6 ml). Subsequently, additional distilled water (70 ml) and 10 mg/ml tRNA (0.1 ml) was added and the entire reaction was extracted through phenol:chloroform:isoamyl alcohol (25:24:1, 100 ml). The aqueous phase was removed, collected and diluted by 5M NaCl (10 ml) and absolute ethanol (-20°C, 250 ml) and centrifuged for 20 minutes at 14,000 x g. The DNA pellet was decanted, resuspended into 70% ethanol (0.5 ml) and centrifuged again for 2 minutes at 14,000 x g. The supernatant was



5 removed and the residue pellet was dried in a speedvac and resuspended  
in distilled water (3 ml). The ligated cDNA/pSST-amy.1 vector DNA was  
chilled on ice to which was added electrocompetent DH10B bacteria (Life  
Tech., 20 ml). The bacteria vector mixture was then electroporated as  
recommended by the manufacturer. Subsequently, SOC media (Life Tech., 1  
10 ml) was added and the mixture was incubated at 37°C for 30 minutes. The  
transformants were then plated onto 20 standard 150 mm LB plates  
containing ampicillin and incubated for 16 hours (37°C). Positive  
colonies were scraped off the plates and the DNA was isolated from the  
bacterial pellet using standard protocols, e.g. CsCl-gradient.

15 The cDNA libraries were screened by hybridization with a  
synthetic oligonucleotide probe:

GGGAGCCGCTCATGAGGAAGTTGGGCCTCATGGACAATGAGATAAAGGTGGCTAAAGCTGAGGCAGCGGG  
(SEQ ID NO:3) based on the EST.

20 Three cDNA clones were sequenced in entirety. The  
overlapping coding regions of the cDNAs were identical except for codon  
410 (using the numbering system for Fig. 1); this position encoded a  
leucine residue (TTG) in both pancreatic cDNAs, and a methionine residue  
(ATG) in the kidney cDNA, possibly due to polymorphism.

25 The entire nucleotide sequence of Apo-2 is shown in Figure 1  
(SEQ ID NO:2). Clone 27868 (also referred to as pRK5-Apo-2 deposited as  
ATCC 209021, as indicated below) contains a single open reading frame  
with an apparent translational initiation site at nucleotide positions  
140-142 [Kozak et al., supra] and ending at the stop codon found at  
nucleotide positions 1373-1375 (Fig. 1; SEQ ID NO:2). The predicted  
30 polypeptide precursor is 411 amino acids long, a type I transmembrane  
protein, and has a calculated molecular weight of approximately 45 kDa.  
Hydropathy analysis (not shown) suggested the presence of a signal  
sequence (residues 1-53), followed by an extracellular domain (residues  
54-182), a transmembrane domain (residues 183-208), and an intracellular  
35 domain (residues 209-411) (Fig. 2A; SEQ ID NO:1). N-terminal amino acid  
sequence analysis of Apo-2-IgG expressed in 293 cells showed that the  
mature polypeptide starts at amino acid residue 54, indicating that the  
actual signal sequence comprises residues 1-53. Apo-2 polypeptide is  
obtained or obtainable by expressing the molecule encoded by the cDNA  
40 insert of the deposited ATCC 209021 vector.

TNF receptor family proteins are typically characterized by

5 the presence of multiple (usually four) cysteine-rich domains in their  
extracellular regions -- each cysteine-rich domain being approximately  
45 amino acids long and containing approximately 6, regularly spaced,  
cysteine residues. Based on the crystal structure of the type 1 TNF  
10 receptor, the cysteines in each domain typically form three disulfide  
bonds in which usually cysteines 1 and 2, 3 and 5, and 4 and 6 are  
paired together. Like DR4, Apo-2 contains two extracellular cysteine-  
rich pseudorepeats (Fig. 2A), whereas other identified mammalian TNFR  
family members contain three or more such domains [Smith et al., Cell,  
76:959 (1994)].

15 The cytoplasmic region of Apo-2 contains a death domain  
(amino acid residues 324-391 shown in Fig. 1; see also Fig. 2A) which  
shows significantly more amino acid sequence identity to the death  
domain of DR4 (64%) than to the death domain of TNFR1 (30%); CD95 (19%);  
or Apo-3/DR3 (29%) (Fig. 2B). Four out of six death domain amino acids  
20 that are required for signaling by TNFR1 [Tartaglia et al., supra] are  
conserved in Apo-2 while the other two residues are semi-conserved (see  
Fig. 2B).

Based on an alignment analysis (using the ALIGN™ computer  
program) of the full-length sequence, Apo-2 shows more sequence identity  
25 to DR4 (55%) than to other apoptosis-linked receptors, such as TNFR1  
(19%); CD95 (17%); or Apo-3 (also referred to as DR3, WSL-1 or TRAMP)  
(29%).

## EXAMPLE 2

### A. Expression of Apo-2 ECD

30 A soluble extracellular domain (ECD) fusion construct was  
prepared. An Apo-2 ECD (amino acid residues 1-184 shown in Figure 1)  
was obtained by PCR and fused to a C-terminal Flag epitope tag (Sigma).  
(The Apo-2 ECD construct included residues 183 and 184 shown in Figure 1  
35 to provide flexibility at the junction, even though residues 183 and 184  
are predicted to be in the transmembrane region). The Flag epitope-  
tagged molecule was then inserted into pRK5, and expressed by transient  
transfection into human 293 cells (ATCC CRL 1573).

40 After a 48 hour incubation, the cell supernatants were  
collected and either used directly for co-precipitation studies (see  
Example 3) or subjected to purification of the Apo-2 ECD-Flag by  
affinity chromatography on anti-Flag agarose beads, according to

5 manufacturer's instructions (Sigma).

#### B. Expression of Apo-2 ECD as an Immunoadhesin

A soluble Apo-2 ECD immunoadhesin construct was prepared. The Apo-2 ECD (amino acids 1-184 shown in Fig. 1) was fused to the hinge and Fc region of human immunoglobulin G<sub>1</sub> heavy chain in pRK5 as described previously [Ashkenazi et al., Proc. Natl. Acad. Sci., 88:10535-10539 (1991)]. The immunoadhesin was expressed by transient transfection into human 293 cells and purified from cell supernatants by protein A affinity chromatography, as described by Ashkenazi et al.,  
15 supra.

#### EXAMPLE 3

##### Immunoprecipitation Assay Showing Binding Interaction Between Apo-2 and Apo-2 Ligand

To determine whether Apo-2 and Apo-2L interact or associate with each other, supernatants from mock-transfected 293 cells or from 293 cells transfected with Apo-2 ECD-Flag (described in Example 2 above) (5 ml) were incubated with 5 µg poly-histidine-tagged soluble Apo-2L [Pitti et al., supra] for 30 minutes at room temperature and then  
25 analyzed for complex formation by a co-precipitation assay.

The samples were subjected to immunoprecipitation using 25 µl anti-Flag conjugated agarose beads (Sigma) or Nickel-conjugated agarose beads (Qiagen). After a 1.5 hour incubation at 4° C, the beads were spun down and washed four times in phosphate buffered saline (PBS). By using anti-Flag agarose, the Apo-2L was precipitated through the Flag-tagged Apo-2 ECD; by using Nickel-agarose, the Apo-2 ECD was precipitated through the His-tagged Apo-2L. The precipitated proteins were released by boiling the beads for 5 minutes in SDS-PAGE buffer, resolved by electrophoresis on 12% polyacrylamide gels, and then  
35 detected by immunoblot with anti-Apo-2L or anti-Flag antibody (2 µg/ml) as described in Marsters et al., J. Biol. Chem., (1997).

The results, shown in Figure 3, indicate that the Apo-2 ECD and Apo-2L can associate with each other.

The binding interaction was further analyzed by purifying Apo-2 ECD from the transfected 293 cell supernatants with anti-Flag beads (see Example 2) and then analyzing the samples on a BIACORE™  
40

5 instrument. The BIAcore™ analysis indicated a dissociation constant (K<sub>d</sub>) of about 1 nM. BIAcore™ analysis also showed that the Apo-2 ECD is not capable of binding other apoptosis-inducing TNF family members, namely, TNF-alpha (Genentech, Inc., Pennica et al., Nature, 312:712 (1984), lymphotoxin-alpha (Genentech, Inc.), or Fas/Apo-1 ligand (Alexis Biochemicals). The data thus shows that Apo-2 is a specific receptor for Apo-2L.

#### EXAMPLE 4

##### Induction of Apoptosis by Apo-2

15 Because death domains can function as oligomerization interfaces, over-expression of receptors that contain death domains may lead to activation of signaling in the absence of ligand [Frazer et al., supra, Nagata et al., supra]. To determine whether Apo-2 was capable of inducing cell death, human 293 cells or HeLa cells (ATCC CCL 2.2) were transiently transfected by calcium phosphate precipitation (293 cells) or electroporation (HeLa cells) with a pRK5 vector or pRK5-based plasmids encoding Apo-2 and/or CrmA. When applicable, the total amount of plasmid DNA was adjusted by adding vector DNA. Apoptosis was assessed 24 hours after transfection by morphology (Fig. 4A); DNA fragmentation (Fig. 4B); or by FACS analysis of phosphatidylserine exposure (Fig. 4C) as described in Marsters et al., Curr. Biol., 6:1669 (1996). As shown in Figs. 4A and 4B, the Apo-2 transfected 293 cells underwent marked apoptosis.

20 For samples assayed by FACS, the HeLa cells were co-transfected with pRK5-CD4 as a marker for transfection and apoptosis was determined in CD4-expressing cells; FADD was co-transfected with the Apo-2 plasmid; the data are means  $\pm$  SEM of at least three experiments, as described in Marsters et al., Curr. Biol., 6:1669 (1996). The caspase inhibitors, DEVD-fmk (Enzyme Systems) or z-VAD-fmk (Research Biochemicals Intl.) were added at 200  $\mu$ M at the time of transfection. As shown in Fig. 4C, the caspase inhibitors CrmA, DEVD-fmk, and z-VAD-fmk blocked apoptosis induction by Apo-2, indicating the involvement of Ced-3-like proteases in this response.

30 FADD is an adaptor protein that mediates apoptosis activation by CD95, TNFR1, and Apo-3/DR3 [Nagata et al., supra], but does not appear necessary for apoptosis induction by Apo-2L [Marsters et

5 al., supra] or by DR4 [Pan et al., supra]. A dominant-negative mutant  
form of FADD, which blocks apoptosis induction by CD95, TNFR1, or Apo-  
3/DR3 [Frazer et al., supra; Nagata et al., supra; Chinnayian et al.,  
supra] did not inhibit apoptosis induction by Apo-2 when co-transfected  
10 signals apoptosis independently of FADD. Consistent with this  
conclusion, a glutathione-S-transferase fusion protein containing the  
Apo-2 cytoplasmic region did not bind to *in vitro* transcribed and  
translated FADD (data not shown).

#### 15 EXAMPLE 5

##### Inhibition of Apo-2L Activity by Soluble Apo-2 ECD

Soluble Apo-2L (0.5 µg/ml, prepared as described in Pitti et  
al., supra) was pre-incubated for 1 hour at room temperature with PBS  
buffer or affinity-purified Apo-2 ECD (5 µg/ml) together with anti-Flag  
20 antibody (Sigma) (1 µg/ml) and added to HeLa cells. After a 5 hour  
incubation, the cells were analyzed for apoptosis by FACS (as above)  
(Fig. 4D).

Apo-2L induced marked apoptosis in HeLa cells, and the  
soluble Apo-2 ECD was capable of blocking Apo-2L action (Fig. 4D),  
25 confirming a specific interaction between Apo-2L and Apo-2. Similar  
results were obtained with the Apo-2 ECD immunoadhesin (Fig. 4D). Dose-  
response analysis showed half-maximal inhibition at approximately 0.3 nM  
Apo-2 immunoadhesin (Fig. 4E).

#### 30 EXAMPLE 6

##### Activation of NF-KB by Apo-2

An assay was conducted to determine whether Apo-2 activates  
NF-KB.

HeLa cells were transfected with pRK5 expression plasmids  
35 encoding full-length native sequence Apo-2, DR4 or Apo-3 and harvested  
24 hours after transfection. Nuclear extracts were prepared and 1 µg of  
nuclear protein was reacted with a <sup>32</sup>P-labelled NF-KB-specific synthetic  
oligonucleotide probe

ATCAGGGACTTTCCGCTGGGGACTTTCCG (SEQ ID NO:4) [see, also, MacKay et al.,  
40 J. Immunol., 153:5274-5284 (1994)], alone or together with a 50-fold

5 excess of unlabelled probe, or with an irrelevant <sup>32</sup>P-labelled synthetic oligonucleotide

AGGATGGGAAGTGTGTGATATATCCTTGAT (SEQ ID NO:5). In some samples, antibody to p65/RelA subunits of NF-KB (1 µg/ml; Santa Cruz Biotechnology) was added. DNA binding was analyzed by an  
10 electrophoretic mobility shift assay as described by Hsu et al., supra; Marsters et al., supra, and MacKay et al., supra.

The results are shown in Fig. 5. As shown in Fig. 5A, upon transfection into HeLa cells, both Apo-2 and DR4 induced significant NF-KB activation as measured by the electrophoretic mobility shift assay;  
15 the level of activation was comparable to activation observed for Apo-3/DR3. Antibody to the p65/RelA subunit of NF-KB inhibited the mobility of the NF-KB probe, implicating p65 in the response to all 3 receptors.

An assay was also conducted to determine if Apo-2L itself can regulate NF-KB activity. HeLa cells or MCF7 cells (human breast  
adenocarcinoma cell line, ATCC HTB 22) were treated with PBS buffer, soluble Apo-2L (Pitti et al., supra) or TNF-alpha (Genentech, Inc., see Pennica et al., Nature, 312:721 (1984)) (1 µg/ml) and assayed for NF-KB activity as above. The results are shown in Fig. 5B. The Apo-2L  
20 induced a significant NF-KB activation in the treated HeLa cells but not in the treated MCF7 cells; the TNF-alpha induced a more pronounced activation in both cell lines. Several studies have disclosed that NF-KB activation by TNF can protect cells against TNF-induced apoptosis [Nagata, supra].

The effects of a NF-KB inhibitor, ALLN (N-acetyl-Leu-Leu-norleucinal) and a transcription inhibitor, cyclohexamide, were also tested. The HeLa cells (plated in 6-well dishes) were preincubated with PBS buffer, ALLN (Calbiochem) (40 µg/ml) or cyclohexamide (Sigma) (50 µg/ml) for 1 hour before addition of Apo-2L (1 µg/ml). After a 5 hour incubation, apoptosis was analyzed by FACS (see Fig. 5C).

35 The results are shown in Fig. 5C. Both ALLN and cyclohexamide increased the level of Apo-2L-induced apoptosis in the HeLa cells. The data indicates that Apo-2L can induce protective NF-KB-dependent genes. The data also indicates that Apo-2L is capable of

5 activating NF-KB in certain cell lines and that both Apo-2 and DR4 may mediate that function.

#### EXAMPLE 7

##### Northern Blot Analysis

10 Expression of Apo-2 mRNA in human tissues was examined by Northern blot analysis. Human RNA blots were hybridized to a 4.6 kilobase <sup>32</sup>P-labelled DNA probe based on the full length Apo-2 cDNA; the probe was generated by digesting the pRK5-Apo-2 plasmid with EcoRI. Human fetal RNA blot MTN (Clontech) and human adult RNA blot MTN-II  
15 (Clontech) were incubated with the DNA probes. Blots were incubated with the probes in hybridization buffer (5X SSPE; 2X Denhardt's solution; 100 mg/mL denatured sheared salmon sperm DNA; 50% formamide; 2% SDS) for 60 hours at 42°C. The blots were washed several times in 2X SSC; 0.05% SDS for 1 hour at room temperature, followed by a 30 minute  
20 wash in 0.1X SSC; 0.1% SDS at 50°C. The blots were developed after overnight exposure.

As shown in Fig. 6, a predominant mRNA transcript of approximately 4.6kb was detected in multiple tissues. Expression was relatively high in fetal and adult liver and lung, and in adult ovary  
25 and peripheral blood leukocytes (PBL), while no mRNA expression was detected in fetal and adult brain. Intermediate levels of expression were seen in adult colon, small intestine, testis, prostate, thymus, pancreas, kidney, skeletal muscle, placenta, and heart. Several adult tissues that express Apo-2, e.g., PBL, ovary, and spleen, have been  
30 shown previously to express DR4 [Pan et al., supra], however, the relative levels of expression of each receptor mRNA appear to be different.

#### EXAMPLE 8

##### Chromosomal Localization of the Apo-2 gene

35 Chromosomal localization of the human Apo-2 gene was examined by radiation hybrid (RH) panel analysis. RH mapping was performed by PCR using a human-mouse cell radiation hybrid panel (Research Genetics) and primers based on the coding region of the Apo-2 cDNA [Gelb et al.,  
40 Hum. Genet., 98:141 (1996)]. Analysis of the PCR data using the Stanford Human Genome Center Database indicates that Apo-2 is linked to

5 the marker D8S481, with an LOD of 11.05; D8S481 is linked in turn to D8S2055, which maps to human chromosome 8p21. A similar analysis of DR4 showed that DR4 is linked to the marker D8S2127 (with an LOD of 13.00), which maps also to human chromosome 8p21.

To Applicants' present knowledge, to date, no other member of the TNFR gene family has been located to chromosome 8.

#### EXAMPLE 9

##### Preparation of Monoclonal Antibodies Specific for Apo-2

Balb/c mice (obtained from Charles River Laboratories) were immunized by injecting 0.5µg/50µl of an Apo-2 ECD immunoadhesin protein (diluted in MPL-TDM adjuvant purchased from Ribi Immunochemical Research Inc., Hamilton, MT) 11 times into each hind foot pad at 3-4 day intervals. The Apo-2 ECD immunoadhesin protein was generated by fusing an extracellular domain sequence of Apo-2 (amino acids 1-184 shown in Fig. 1) to the hinge and Fc region of human immunoglobulin G<sub>1</sub> heavy chain in pRK5 as described previously [Ashkenazi et al., Proc. Natl. Acad. Sci., 88:10535-10539 (1991)]. The immunoadhesin protein was expressed by transient transfection into human 293 cells and purified from cell supernatants by protein A affinity chromatography, as described by Ashkenazi et al., supra (See also Example 2B above).

Three days after the final boost, popliteal lymph nodes were removed from the mice and a single cell suspension was prepared in DMEM media (obtained from Biowhitakker Corp.) supplemented with 1% penicillin-streptomycin. The lymph node cells were then fused with murine myeloma cells P3X63AgU.1 (ATCC CRL 1597) using 35% polyethylene glycol and cultured in 96-well culture plates. Hybridomas resulting from the fusion were selected in HAT medium. Ten days after the fusion, hybridoma culture supernatants were screened in an ELISA to test for the presence of monoclonal antibodies binding to the Apo-2 ECD immunoadhesin protein.

In the ELISA, 96-well microtiter plates (Maxisorb; Nunc, Kamstrup, Denmark) were coated by adding 50 µl of 2 µg/ml goat anti-human IgG Fc (purchased from Cappel Laboratories) in PBS to each well and incubating at 4°C overnight. The plates were then washed three times with wash buffer (PBS containing 0.05% Tween 20). The wells in the microtiter plates were then blocked with 50 µl of 2.0% bovine serum



5 albumin in PBS and incubated at room temperature for 1 hour. The plates were then washed again three times with wash buffer.

After the washing step, 50  $\mu$ l of 0.4  $\mu$ g/ml Apo-2 ECD immunoadhesin protein (as described above) in assay buffer was added to each well. The plates were incubated for 1 hour at room temperature on a shaker apparatus, followed by washing three times with wash buffer.

Following the wash steps, 100  $\mu$ l of the hybridoma supernatants or purified antibody (using Protein A-sepharose columns) (1  $\mu$ g/ml) was added to designated wells in the presence of CD4-IgG. 100  $\mu$ l of P3X63AgU.1 myeloma cell conditioned medium was added to other designated wells as controls. The plates were incubated at room temperature for 1 hour on a shaker apparatus and then washed three times with wash buffer.

Next, 50  $\mu$ l HRP-conjugated goat anti-mouse IgG Fc (purchased from Cappel Laboratories), diluted 1:1000 in assay buffer (0.5% bovine serum albumin, 0.05% Tween-20, 0.01% Thimersol in PBS), was added to each well and the plates incubated for 1 hour at room temperature on a shaker apparatus. The plates were washed three times with wash buffer, followed by addition of 50  $\mu$ l of substrate (TMB microwell peroxidase substrate, Kirkegaard & Perry, Gaithersburg, MD) to each well and incubation at room temperature for 10 minutes. The reaction was stopped by adding 50  $\mu$ l of TMB 1-component stop solution (diethyl glycol, Kirkegaard & Perry) to each well, and absorbance at 450 nm was read in an automated microtiter plate reader.

Of the hybridoma supernatants screened in the ELISA, 22 supernatants tested positive (calculated as approximately 4 times above background). The supernatants testing positive in the ELISA were further analyzed by FACS analysis using 9D cells (a human B lymphoid cell line expressing Apo-2; Genentech, Inc.) and FITC-conjugated goat anti-mouse IgG. For this analysis, 25  $\mu$ l of cells suspended (at  $4 \times 10^6$  cells/ml) in cell sorter buffer (PBS containing 1% FCS and 0.02%  $\text{NaN}_3$ ) were added to U-bottom microtiter wells, mixed with 100  $\mu$ l of culture supernatant or purified antibody (purified on Protein A-sepharose columns) (10  $\mu$ g /ml) in cell sorter buffer, and incubated for 30 minutes on ice. The cells were then washed and incubated with 100  $\mu$ l FITC-

5 conjugated goat anti-mouse IgG for 30 minutes at 4°C. Cells were then washed twice, resuspended in 150 µl of cell sorter buffer and then analyzed by FACScan (Becton Dickinson, Mountain View, CA). FACS analysis showed 8/22 supernatants were positive for anti-Apo-2 antibodies.

10 Figure 7 shows the FACS staining of 9D cells incubated with one of the Apo-2 antibodies, referred to as 3F11.39.7. As shown in Figure 7, the 3F11.39.7 antibody recognizes the Apo-2 receptor expressed in 9D cells.

#### 15 EXAMPLE 10

##### Assay for Ability of Apo-2 Abs to Agonistically induce Apoptosis

20 Hybridoma supernatants and purified antibodies (as described in Example 9 above) were tested for activity to induce Apo-2 mediated 9D cell apoptosis. The 9D cells ( $5 \times 10^5$  cells/0.1ml) were incubated with varying concentrations of antibodies in 100 µl complete RPMI media at 4°C for 15 minutes. The cells were then incubated for 5 minutes at 37°C and 10 µg of goat anti-mouse IgG Fc antibody (Cappel Laboratories) in 300 µl of complete RPMI was added to some of the cell samples. At this point, the cells were incubated overnight at 37°C and in the presence of 7% CO<sub>2</sub>. The cells were then harvested and washed once with PBS. The viability of the cells was determined by staining of FITC-annexin V binding to phosphatidylserine according to manufacturer recommendations (Clontech). The cells were washed in PBS and resuspended in 200 µl binding buffer. Ten µl of annexin-V-FITC (1 µg/ml) and 10 µl of propidium iodide were added to the cells. After incubation for 15 minutes in the dark, the 9D cells were analyzed by FACS.

30 As shown in Figure 8, the 3F11.39.7 antibody (in the absence of the goat anti-mouse IgG Fc) induced apoptosis in the 9D cells as compared to the control antibodies. Agonistic activity, however, was enhanced by Apo-2 receptor cross-linking in the presence of the goat anti-mouse IgG Fc (see Figure 9). This enhanced apoptosis (Figure 9) by the combination of antibodies is comparable to the apoptotic activity of Apo-2L in 9D cells (data not shown).

Assay for Antibody Ability to Block Apo-2 ligand-induced Apoptosis

Hybridoma supernatants and purified antibodies (as described in Example 9 above) were tested for activity to block Apo-2 ligand induced 9D cell apoptosis. The 9D cells ( $5 \times 10^5$  cells/0.1ml) were suspended in complete RPMI media (RPMI plus 10%FCS, glutamine, nonessential amino acids, penicillin, streptomycin, sodium pyruvate) and placed into individual Falcon 2052 tubes. Cells were then incubated with 10  $\mu$ g of antibodies in 200  $\mu$ l media for 15 minutes on ice. 0.2 ml of Apo-2 ligand (2.5  $\mu$ g/ml) (soluble His-tagged Apo-2L prepared as described in WO 97/25428; see also Pitti et al., *supra*) was suspended into complete RPMI media, and then added into the tubes containing the 9D cells. The 9D cells were incubated overnight at 37°C and in the presence of 7% CO<sub>2</sub>. The incubated cells were then harvested and washed once with PBS. The viability of the cells was determined by staining of FITC-annexin V binding to phosphatidylserine according to manufacturer recommendations (Clontech). Specifically, the cells were washed in PBS and resuspended in 200  $\mu$ l binding buffer. Ten  $\mu$ l of annexin-V-FITC (1  $\mu$ g/ml) and 10  $\mu$ l of propidium iodide were added to the cells. After incubation for 15 minutes in the dark, the 9D cells were analyzed by FACS.

The results are shown in Figure 10. Since 9D cells express more than one receptor for Apo-2L, Apo-2L can induce apoptosis in the 9D cells by interacting with either Apo-2 or the DR4 receptor. Thus, to detect any blocking activity of the Apo-2 antibodies, the interaction between DR4 and Apo-2L needed to be blocked. In combination with the anti-DR4 antibody, 4H6.17.8 (ATCC HB-12455), the Apo-2 antibody 3F11.39.7 was able to block approximately 50% of apoptosis induced by Apo-2L. The remaining approximately 50% apoptotic activity is believed to be due to the agonistic activities of these two antibodies by themselves, as shown in Figure 10. Accordingly, it is believed that the 3F11.39.7 antibody is a blocking Apo-2 antibody.

EXAMPLE 12ELISA Assay to Test Binding of Apo-2 Antibodies to OtherApo-2 Ligand Receptors

An ELISA was conducted to determine if the monoclonal antibody described in Example 9 was able to bind other known Apo-2L receptors beside Apo-2. Specifically, the 3F11.39.7 antibody was tested for binding to DR4 [Pan et al., supra], DcR1 [Sheridan et al., supra], and DcR2 [Marsters et al., Curr. Biol., 7:1003-1006 (1997)]. The ELISA was performed essentially as described in Example 9 above.

The results are shown in Figure 11. The Apo-2 antibody 3F11.39.7 bound to Apo-2. The 3F11.39.7 antibody also showed some cross-reactivity to DR4, but not to DcR1 or DcR2.

EXAMPLE 13Antibody Isotyping

The isotype of the 3F11.39.7 antibody (as described above) was determined by coating microtiter plates with isotype specific goat anti-mouse Ig (Fisher Biotech, Pittsburgh, PA) overnight at 4°C. The plates were then washed with wash buffer (as described in Example 9 above). The wells in the microtiter plates were then blocked with 200 µl of 2% bovine serum albumin and incubated at room temperature for one hour. The plates were washed again three times with wash buffer. Next, 100 µl of 5 µg/ml of purified 3F11.39.7 antibody was added to designated wells. The plates were incubated at room temperature for 30 minutes and then 50 µl HRP-conjugated goat anti-mouse IgG (as described above) was added to each well. The plates were incubated for 30 minutes at room temperature. The level of HRP bound to the plate was detected using HRP substrate as described above.

The isotyping analysis showed that the 3F11.39.7 antibody is an IgG1 antibody.

\* \* \* \* \*

40 Deposit of Material

The following materials have been deposited with the American

5 Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, USA  
(ATCC):

<u>Material</u>	<u>ATCC Dep. No.</u>	<u>Deposit Date</u>
pRK5-Apo-2	209021	May 8, 1997
3F11.39.7	HB-12456	January 13, 1998

10

15 This deposit was made under the provisions of the Budapest  
Treaty on the International Recognition of the Deposit of Microorganisms  
for the Purpose of Patent Procedure and the Regulations thereunder  
(Budapest Treaty). This assures maintenance of a viable culture of the  
deposit for 30 years from the date of deposit. The deposit will be made  
available by ATCC under the terms of the Budapest Treaty, and subject to  
an agreement between Genentech, Inc. and ATCC, which assures permanent  
and unrestricted availability of the progeny of the culture of the  
20 deposit to the public upon issuance of the pertinent U.S. patent or upon  
laying open to the public of any U.S. or foreign patent application,  
whichever comes first, and assures availability of the progeny to one  
determined by the U.S. Commissioner of Patents and Trademarks to be  
entitled thereto according to 35 USC Section 122 and the Commissioner's  
25 rules pursuant thereto (including 37 CFR Section 1.14 with particular  
reference to 886 OG 638).

30 The assignee of the present application has agreed that if a  
culture of the materials on deposit should die or be lost or destroyed  
when cultivated under suitable conditions, the materials will be  
promptly replaced on notification with another of the same.  
Availability of the deposited material is not to be construed as a  
license to practice the invention in contravention of the rights granted  
under the authority of any government in accordance with its patent  
laws.

35 The foregoing written specification is considered to be  
sufficient to enable one skilled in the art to practice the invention.  
The present invention is not to be limited in scope by the construct  
deposited, since the deposited embodiment is intended as a single  
illustration of certain aspects of the invention and any constructs that  
40 are functionally equivalent are within the scope of this invention. The  
deposit of material herein does not constitute an admission that the  
written description herein contained is inadequate to enable the



## (1) GENERAL INFORMATION:

(i) APPLICANT: Ashkenazi, Avi  
Chuntharapai, Anon  
Kim, Kyung Jin

(ii) TITLE OF INVENTION: Apo-2 RECEPTOR

(iii) NUMBER OF SEQUENCES: 5

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(B) STREET: 1 DNA Way  
(C) CITY: South San Francisco  
(D) STATE: California  
(E) COUNTRY: USA  
(F) ZIP: 94080

## (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: 3.5 inch, 1.44 Mb floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: WinPatin (Genentech)

## (vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:  
(B) FILING DATE:  
(C) CLASSIFICATION:

## (viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Marschang, Diane L.  
(B) REGISTRATION NUMBER: 35,600  
(C) REFERENCE/DOCKET NUMBER: PR1101P1

5 (ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 650/225-5416

(B) TELEFAX: 650/952-9881

(C) TELEX: 910/371-7168

10 (2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 411 amino acids

(B) TYPE: Amino Acid

15 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Met Glu Gln Arg Gly Gln Asn Ala Pro Ala Ala Ser Gly Ala Arg  
 1 5 10 15  
 Lys Arg His Gly Pro Gly Pro Arg Glu Ala Arg Gly Ala Arg Pro  
 20 25 30  
 Gly Leu Arg Val Pro Lys Thr Leu Val Leu Val Val Ala Ala Val  
 35 40 45  
 Leu Leu Leu Val Ser Ala Glu Ser Ala Leu Ile Thr Gln Gln Asp  
 50 55 60  
 Leu Ala Pro Gln Gln Arg Ala Ala Pro Gln Gln Lys Arg Ser Ser  
 65 70 75  
 Pro Ser Glu Gly Leu Cys Pro Pro Gly His His Ile Ser Glu Asp  
 80 85 90  
 Gly Arg Asp Cys Ile Ser Cys Lys Tyr Gly Gln Asp Tyr Ser Thr  
 95 100 105  
 His Trp Asn Asp Leu Leu Phe Cys Leu Arg Cys Thr Arg Cys Asp  
 110 115 120





5

Phe Ala Asp Leu Val Pro Phe Asp Ser Trp Glu Pro Leu Met Arg  
320 325 330

10

Lys Leu Gly Leu Met Asp Asn Glu Ile Lys Val Ala Lys Ala Glu  
335 340 345

Ala Ala Gly His Arg Asp Thr Leu Tyr Thr Met Leu Ile Lys Trp  
350 355 360

15

Val Asn Lys Thr Gly Arg Asp Ala Ser Val His Thr Leu Leu Asp  
365 370 375

Ala Leu Glu Thr Leu Gly Glu Arg Leu Ala Lys Gln Lys Ile Glu  
380 385 390

20

Asp His Leu Leu Ser Ser Gly Lys Phe Met Tyr Leu Glu Gly Asn  
395 400 405

25

Ala Asp Ser Ala Xaa Ser  
410 411

(2) INFORMATION FOR SEQ ID NO:2:

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- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 1799 base pairs
  - (B) TYPE: Nucleic Acid
  - (C) STRANDEDNESS: Single
  - (D) TOPOLOGY: Linear

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CCCACGCGTC CGCATAAATC AGCACGCGGC CGGAGAACCC CGCAATCTCT 50

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GCGCCACAA AATACACCGA CGATGCCCGA TCTACTTTAA GGGCTGAAAC 100

CCACGGGCCT GAGAGACTAT AAGAGCGTTC CCTACCGCC ATG GAA 145

5

Met Glu

1

CAA CGG GGA CAG AAC GCC CCG GCC GCT TCG GGG GCC CGG 184

Gln Arg Gly Gln Asn Ala Pro Ala Ala Ser Gly Ala Arg

10

5

10

15

AAA AGG CAC GGC CCA GGA CCC AGG GAG GCG CGG GGA GCC 223

Lys Arg His Gly Pro Gly Pro Arg Glu Ala Arg Gly Ala

20

25

15

AGG CCT GGG CTC CGG GTC CCC AAG ACC CTT GTG CTC GTT 262

Arg Pro Gly Leu Arg Val Pro Lys Thr Leu Val Leu Val

30

35

40

20

GTC GCC GCG GTC CTG CTG TTG GTC TCA GCT GAG TCT GCT 301

Val Ala Ala Val Leu Leu Leu Val Ser Ala Glu Ser Ala

45

50

25

CTG ATC ACC CAA CAA GAC CTA GCT CCC CAG CAG AGA GCG 340

Leu Ile Thr Gln Gln Asp Leu Ala Pro Gln Gln Arg Ala

55

60

65

30

GCC CCA CAA CAA AAG AGG TCC AGC CCC TCA GAG GGA TTG 379

Ala Pro Gln Gln Lys Arg Ser Ser Pro Ser Glu Gly Leu

70

75

80

TGT CCA CCT GGA CAC CAT ATC TCA GAA GAC GGT AGA GAT 418

Cys Pro Pro Gly His His Ile Ser Glu Asp Gly Arg Asp

85

90

35

TGC ATC TCC TGC AAA TAT GGA CAG GAC TAT AGC ACT CAC 457

Cys Ile Ser Cys Lys Tyr Gly Gln Asp Tyr Ser Thr His

95

100

105

40

TGG AAT GAC CTC CTT TTC TGC TTG CGC TGC ACC AGG TGT 496

Trp Asn Asp Leu Leu Phe Cys Leu Arg Cys Thr Arg Cys

110

115

75

35020-674009

5

GAT TCA GGT GAA GTG GAG CTA AGT CCC TGC ACC ACG ACC 535  
 Asp Ser Gly Glu Val Glu Leu Ser Pro Cys Thr Thr Thr  
 120 125 130

10 AGA AAC ACA GTG TGT CAG TGC GAA GAA GGC ACC TTC CGG 574  
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15 GAA GAA GAT TCT CCT GAG ATG TGC CGG AAG TGC CGC ACA 613  
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 150 155

20 GGG TGT CCC AGA GGG ATG GTC AAG GTC GGT GAT TGT ACA 652  
 Gly Cys Pro Arg Gly Met Val Lys Val Gly Asp Cys Thr  
 160 165 170

25 CCC TGG AGT GAC ATC GAA TGT GTC CAC AAA GAA TCA GGC 691  
 Pro Trp Ser Asp Ile Glu Cys Val His Lys Glu Ser Gly  
 175 180

5 ATC ATC ATA GGA GTC ACA GTT GCA GCC GTA GTC TTG ATT 730  
 Ile Ile Ile Gly Val Thr Val Ala Ala Val Val Leu Ile  
 185 190 195

10 GTG GCT GTG TTT GTT TGC AAG TCT TTA CTG TGG AAG AAA 769  
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 200 205 210

15 GTC CTT CCT TAC CTG AAA GGC ATC TGC TCA GGT GGT GGT 808  
 Val Leu Pro Tyr Leu Lys Gly Ile Cys Ser Gly Gly Gly  
 215 220

20 GGG GAC CCT GAG CGT GTG GAC AGA AGC TCA CAA CGA CCT 847  
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 225 230 235

25 GGG GCT GAG GAC AAT GTC CTC AAT GAG ATC GTG AGT ATC 886  
 Gly Ala Glu Asp Asn Val Leu Asn Glu Ile Val Ser Ile  
 240 245

30 TTG CAG CCC ACC CAG GTC CCT GAG CAG GAA ATG GAA GTC 925  
 Leu Gln Pro Thr Gln Val Pro Glu Gln Glu Met Glu Val  
 250 255 260

35 CAG GAG CCA GCA GAG CCA ACA GGT GTC AAC ATG TTG TCC 964  
 Gln Glu Pro Ala Glu Pro Thr Gly Val Asn Met Leu Ser  
 265 270 275

40 CCC GGG GAG TCA GAG CAT CTG CTG GAA CCG GCA GAA GCT 1003  
 Pro Gly Glu Ser Glu His Leu Leu Glu Pro Ala Glu Ala  
 280 285

GAA AGG TCT CAG AGG AGG AGG CTG CTG GTT CCA GCA AAT 1042  
 Glu Arg Ser Gln Arg Arg Arg Leu Leu Val Pro Ala Asn  
 290 295 300

GAA GGT GAT CCC ACT GAG ACT CTG AGA CAG TGC TTC GAT 1081  
 Glu Gly Asp Pro Thr Glu Thr Leu Arg Gln Cys Phe Asp

5

305

310

GAC TTT GCA GAC TTG GTG CCC TTT GAC TCC TGG GAG CCG 1120  
 Asp Phe Ala Asp Leu Val Pro Phe Asp Ser Trp Glu Pro  
 315 320 325

10

CTC ATG AGG AAG TTG GGC CTC ATG GAC AAT GAG ATA AAG 1159  
 Leu Met Arg Lys Leu Gly Leu Met Asp Asn Glu Ile Lys  
 330 335 340

15

GTG GCT AAA GCT GAG GCA GCG GGC CAC AGG GAC ACC TTG 1198  
 Val Ala Lys Ala Glu Ala Ala Gly His Arg Asp Thr Leu  
 345 350

20

TAC ACG ATG CTG ATA AAG TGG GTC AAC AAA ACC GGG CGA 1237  
 Tyr Thr Met Leu Ile Lys Trp Val Asn Lys Thr Gly Arg  
 355 360 365

25

GAT GCC TCT GTC CAC ACC CTG CTG GAT GCC TTG GAG ACG 1276  
 Asp Ala Ser Val His Thr Leu Leu Asp Ala Leu Glu Thr  
 370 375

30

CTG GGA GAG AGA CTT GCC AAG CAG AAG ATT GAG GAC CAC 1315  
 Leu Gly Glu Arg Leu Ala Lys Gln Lys Ile Glu Asp His  
 380 385 390

TTG TTG AGC TCT GGA AAG TTC ATG TAT CTA GAA GGT AAT 1354  
 Leu Leu Ser Ser Gly Lys Phe Met Tyr Leu Glu Gly Asn  
 395 400 405

366020"6T42009

5 GCA GAC TCT GCC WTG TCC TAAGTGTG ATTCTCTTCA GGAAGTGAGA 1400  
Ala Asp Ser Ala Xaa Ser  
410 411

10 CCTTCCTGG TTTACCTTTT TTCTGGAAAA AGCCCACTG GACTCCAGTC 1450  
AGTAGGAAAG TGCCACAATT GTCACATGAC CGGTACTGGA AGAAACTCTC 1500  
CCATCCAACA TCACCCAGTG GATGGAACAT CCTGTAACCTT TTCACTGCAC 1550

15 TTGGCATTAT TTTTATAAGC TGAATGTGAT AATAAGGACA CTATGGAAAT 1600  
GTCTGGATCA TTCCGTTTGT GCGTACTTTG AGATTGGTT TGGGATGTCA 1650  
TTGTTTTTAC AGCACTTTTT TATCCTAATG TAAATGCTTT ATTTATTTAT 1700

20 TTGGGCTACA TTGTAAGATC CATCTACAAA AAAAAAAAAA AAAAAAAG 1750  
GGCGGCCGCG ACTCTAGAGT CGACCTGCAG AAGCTTGGCC GCCATGGCC 1799

(2) INFORMATION FOR SEQ ID NO:3:

- 25 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 70 base pairs  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
30 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

35 GGGAGCCGCT CATGAGGAAG TTGGGCCTCA TGGACAATGA GATAAAGGTG 50  
GCTAAAGCTG AGGCAGCGGG 70

5 (2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 base pairs

(B) TYPE: Nucleic Acid

10 (C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

15

ATCAGGGACT TTCCGCTGGG GACTTTCCG 29

(2) INFORMATION FOR SEQ ID NO:5:

20

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AGGATGGGAA GTGTGTGATA TATCCTTGAT 30

30



What is claimed is:

1. An antibody which specifically binds to Apo-2.
2. The antibody of claim 1 which is a monoclonal antibody.
3. The antibody of claim 1 which is an agonist antibody.
4. The antibody of claim 1 which is a blocking antibody.
5. The antibody of claim 1 which is a chimeric antibody.
6. A hybridoma cell line which produces the antibody of claim 2.
7. The antibody of claim 2 having the biological characteristics of the monoclonal antibody produced by the hybridoma cell line deposited under American Type Culture Collection Accession Number ATCC HB-12456.
8. The antibody of claim 2 wherein the antibody binds to the same epitope as the epitope to which the monoclonal antibody produced by the hybridoma cell line deposited under American Type Culture Collection Accession Number ATCC HB-12456 binds.
9. The hybridoma cell line deposited under American Type Culture Collection Accession Number HB-12456.
10. The monoclonal antibody produced by the hybridoma cell line deposited under American Type Culture Collection Accession Number ATCC HB-12456.
11. An isolated nucleic acid encoding the Apo-2 antibody of claim 1.
12. A composition comprising the antibody of claim 1 and a carrier.
13. The composition of claim 12 wherein said carrier is a pharmaceutically acceptable carrier.
14. A method of inducing apoptosis in mammalian cells comprising

exposing mammalian cells to an effective amount of an Apo-2 agonist antibody.

15. An article of manufacture, comprising a container and a composition contained within the container, wherein the composition includes an Apo-2 antibody.

16. The article of manufacture of claim 15 further comprising instructions for using the Apo-2 antibody *in vivo* or *in vitro*.

17. A dimeric molecule comprising an Apo-2 antibody linked to a heterologous immunoglobulin.

18. A homodimeric molecule comprising a first Apo-2 antibody and a second Apo-2 antibody.

Abstract of the Disclosure

Novel polypeptides, designated Apo-2, which are capable of modulating apoptosis are provided. Compositions including Apo-2  
10 chimeras, nucleic acid encoding Apo-2, and antibodies to Apo-2 are also provided.

6007419.020998

Fig. 1

1	CCCACGGGTC	CGCATAAATC	AGCAGCGGGC	CGGAGAAACC	CGCAATCTCT	GGCCCCACAA	AATACACCGA	CGATGCCCGA	TCTACTTTAA	GGGCTGAAAC
	GGGTGGCGAG	CGGTATTTAG	TCGTGGCGCG	GCCTCTTGGG	GGCTTAGAGA	CGCGGTGTTT	TTATGTGGCT	GCTACGGGCT	AGATGAAATT	CCCGACTTTG
101	CCACGGGCGT	GAGAGACTAT	AAGAGCGTTC	CCTACCGCCA	TGGAACAACG	GGGACAGAAC	GCCCCGGCGG	CTTCGGGGGG	CCGAAAAAGG	CACGGCCCCAG
	GGTGCCCGGA	CTCTCTGATA	TTCTCGCAAG	GGATGGCGGT	ACCTTGTTGC	CCCTGTCTTG	CGGGCCCGGC	GAAGCCCCCG	GGCCTTTTCC	GTGCCGGGTC
1										
201	GACCCAGGGA	GGCGGGGGA	GCCAGGCGTG	GGTCCGGGT	CCCCAAGACC	CTTGTGCTCG	TTGTGCGCGC	GGTCTGCTG	TTGGTCTCAG	CTGAGTCTGC
	CTGGTCCCT	CCGCGCCCT	CGGTCCGGAC	CCGAGGCCCA	GGGTCTCTGG	GAACAGCGAG	AACAGCGGCG	CCAGGACGAC	AACAGAGTC	GACTCAGACG
22	ProArgG1	uAlaArgGly	AlaArgProG	lyLeuArgVa	lProLysThr	LeuValLeuV	alValAlaAl	avalLeuLeu	LeuValSera	laGluserAla
301	TCTGATCACC	CAACAAGACC	TAGTCCCCCA	GCAGAGAGCG	CCCCCACAAAC	AAAAGAGGTC	CAGCCCCCTCA	GAGGGATTGT	GTCCACCTGG	ACACCATATC
	AGACTACTGG	GTTGTTCTGG	ATCGAGGGGT	CGTCTCTCGC	CGGGGTGTTG	TTTTCTCCAG	GTCCGGGAGT	CTCCCTAACA	CAGGTGGACC	TGTGGTATAG
55	LeuileThr	GlnGlnAspL	euAlaProG1	nGlnArgAla	AlaProGlnG	lnLysArgSe	rSerProSer	GlUGlyLeuC	ysProProG1	yHishisile
401	TCAGAAAGAG	GTAGAGATTG	CATCTCCTGC	AAATATGGAC	AGGACTATAG	CACCTACTGG	AATGACCTCC	TTTTCTGCTT	GGCTGCACC	AGGTGTGATT
	AGTCTTCTGC	CATCTCTAAC	GTAGAGGACG	TTTATACCTG	TCCTGATATC	GTGAGTGACC	TTACTGGAGG	AAAAGACGAA	CGCGACGTGG	TCCACACTAA
88	SerGluAspG	lyArgAspCy	sileSerCys	lystyrglyG	lnAspTyise	rThrHistrp	AsnAspLeuL	eupheCysLe	uArgCysThr	ArgCysAspSer
501	CAGGTGAAGT	GGAGCTAAGT	CCCTGCACCA	CGACCAGAAA	CACAGTGTGT	CAGTGCGAAG	AAGGCACCTT	CCGGAAAGAA	GATTCTCCTG	AGATGTGCCG
	GTCCACTTCA	CCTCGATTCA	GGGACGTGGT	GCTGTCTTTT	GTGTACACAA	GTACAGCTTC	TTCCGTGGAA	GGCCTTCTT	CTAAGAGGAC	TCTACACGGC
122	GlyGluVa	lgluLeuSer	ProCysThrt	hrThrArgAs	nThrValCys	GlnCysGluG	luglyThrPh	eArgGluGlu	AspSerProG	luMetCysArg
601	GAAGTGCCGC	ACAGGGTGTC	CCAGAGGGAT	GGTCAAGGTC	GGTGATTGTA	CACCCCTGGAG	TGACATCGAA	TGTGTCCACA	AAGAATCAGG	CATCATCATA
	CTTCACGGCG	TGTCCACACG	GGTCTCCCTA	CCAGTTCCAG	CCACTAACAT	GTGGGACCTC	ACTGTAGCTT	ACACAGGTGT	TTCTTAGTCC	GTAGTAGTAT
155	LysCysArg	ThrGlyCysP	roArgGlyMe	tValLysVal	GlyAspCyst	hrProTrpSe	rAspIleGlu	CysvalHisL	ysGluSerG1	yIleIleile
701	GGAGTCACAG	TTGCAGCCGT	AGTCTTGATT	GTGGCTGTGT	TTGTTTGCRA	GTCTTTACTG	TGGAAGAAAG	TCCTTCTCTA	CCTGAAAGGC	ATCTGCTCAG
	CCTCAGTGTC	AACGTGGGCA	TCAGAACTAA	CACCGACACA	AACAAACGTT	CAGAAATGAC	ACCTTCTTTC	AGGAAGGAAT	GGACTTCCG	TAGACGAGTC
188	GlyValThrV	alAlaAlaVa	lValleuile	ValAlaValP	heValCysLy	sserLeuLeu	TriPLysLysV	alLeuProTy	rLeuLysGly	IleCysSerGly
801	GTGGTGCTGG	GGACCCCTGAG	CGTGTGGACA	GAAGCTCACA	ACGACCTGGG	GCTGAGGACA	ATGTCTCTCA	TGAGATCGTG	AGTATCTTGC	AGCCCCACCCA
	CACCAACCACC	CCTGGGACTC	GCACACCTGT	CTTCGAGTGT	TGCTGGACCC	CGACTCCTGT	TACAGGAGTT	ACTCTAGCAC	TCATAGAACG	TCGGGTGGGT
222	GlyGlyG1	yAspProGlu	ArgValAspA	rgSerSerG1	nArgProGly	AlaGluAspA	snValLeuAs	ngluileVal	SerileLeug	lnProThrGln
901	GGTCCCTGAG	CAGGAAATGG	AAGTCCAGGA	GCCAGCAGAG	CCAAACAGGTG	TCAACATGTT	GTCCCCCGGG	GAGTCAGAGC	ATCTGCTTGA	ACCGGCAGAA
	CCAGGGACTC	GTCCTTTACC	TTACAGTCTC	CGGTGCTCTC	GGTTGTCCAC	AGTTGTACAA	CAGGGGGCCC	CTCAGTCTCG	TAGACGACCT	TGGCCGTCTT
255	ValProGlu	GlnGluMetG	luValGlnG1	uProAlaGlu	ProThrGlyV	alasnMetLe	userProGly	GluserGluH	isLeuLeuG1	uProAlaGlu
1001	GCTGAAAGGT	CTCAGAGGAG	GAGGCTGCTG	GTTCCAGCAA	ATGAAGGTGA	TCCCACCTGAG	ACTCTGAGAC	AGTGTCTCGA	TGACTTTGCA	GACTTGGTGC
	CGACTTTTCCA	GAGTCTCTC	CTCCGACGAC	CAAGGTCTGT	TACTTCCACT	AGGTGACTC	TGAGACTCTG	TCACGAAGCT	ACTGAAACGT	CTGAACCCAG
288	AlaGluArgs	erGlnArgAr	gArgLeuLeu	ValProAlaA	snGluGlyAs	pProThrGlu	ThrLeuArgG	lnCysPheAs	pAspPheAla	AspLeuValPro

1101 CCTTTGACTC CTGGGAGCG CTCATGAGGA AGTTGGGCCI CATGACACAT GAGATTAAGC TGGCTAAAGC TGAGGCAGCG GGCCACAGGG ACACCTTGTA  
GAAACTGAG GACCTCGGC GAGTACTCCT TCAACCCGGA GTACCTGTTA CTCATTCC ACCGATTTCG ACTCGTCCG CCGGTGTCCT TGTGGAACAT  
322 Pheaspse rTrpGluPro LeuMetArgL ysLeuclyle uMetaspasn Gluilelysv alalaLysal aglualaala GlyHisArga spThrLeuTyr  
1201 CACGATGCTG ATAAAGTGGG TCNACNAAAC CGGGCGAGAT GCCTCTGTCC ACACCCCTGT GGATGCCCTTG GAGACGCTGG GAGAGAGACT TGCCAAAGCAG  
GTGCTACGAC TATTTACCC AGTTGTTTTG GCCCGCTCTA CGGAGACAGG TGTGGGACGA CCTACGGAAC CTCTGCGACC CTCTCTCTGA ACGTTTCGTC  
355 ThrMetLeu IleLysTrpv alaasnLysTh rGlyArgasp Alaservalh isthrLeule uAspAlaLeu GluthrLeug lyGluuArgLe uAlaLysGln  
1301 AAGATTGAGG ACCACTTGTG GAGCTCTGGA AAGTTCAATG TAATGCAGAC TCTGCCWTGT CCTAAGTGTG ATTCTCTTCA GGAAGTGAGA  
TTCTAACTCC TGGTGAACAA CTCGAGACCT TTCAAGTACA TAGATCTTCC ATTACGTCTG AGACGGGAACA GGATTTCACAC TRAGAGAAAGT CCTTCACTCT  
388 LysIleGluA sphisLeule userSergly LysPheMetT yrLeuGluGl yAsnAlaasp SerAlaXqq S erOC\*  
1401 CCTTCCCTGG TTTACCTTTT TTCTGGAAAA AGCCCACTG GACTCCAGTC AGTAGGAAAG TGCCACRAAT GTACATGAC CCGTACTGGA AGAAACTCTC  
GGNAGGGACC AAATGGAAAA AAGACCTTTT TCGGGTTGAC CTGAGGTCAG TCATCCITTC ACGGTGTTAA CAGTGTACTG GCCATGACCT TCTTTGAGAG  
1501 CCATCCAAAC TCACCCAGTG GATGGAACAT CCTGTAACTT TTCACITGCAC TTGGCATTAT TTTTATAAGC TGAATGTGAT AATAAGGACA CTATGGAAAT  
GGTAGGTTGT AGTGGGTCAC CTACCTTGTA GGACATTGAA AAGTGACGTG AACCGTAATA AAAATATTTC ACTTACACTA TTATTCCTGT GATACCTTTA  
1601 GTCTGGATCA TTCCGTTTGT GCGTACTTTG AGATTGGTT AGATTGGTT TGGGATGTCA TTGTTTTTCAC AGCACTTTT TATCCTAATG TAAATGCTTT ATTTATTTAT  
CAGACCTAGT AAGGCAAAAC CGCATGAAAC TCTAAACCAA ACCCTACAGT AACAAAAAGTG TCGTGAAAAA ATAGGATTAC ATTTACGAAA TAAATAAATA  
1701 TTGGGCTACA TTGTAAGATC CATCTACAAA AAAAAAATAA GCGCGCCGCG ACTCTAGAGT CGACCTGCAG AAGCTTGGCC GCCATGGCC  
AACCCGATGT AACATTCTAG GTAGATGTTT TTTTTTTTTT TTTTTTTTTT CCGCCGCGCG TGAGATCTCA GCTGGACGTC TTCGAAACCGG CCGTACCGG

Fig. 1 (cont.)

Fig. 2 A

1 MEORGONAPAAAGARKRHGPGPREARGARPGRLVPKTLVLVVAALLLVSAESALITQQD  
61 LAPQORAAPOQKRSSPSEGLCPGHHISEDGRDCISCKYQDYSTHWNDDLFLRCRTRCD  
121 SGEVELSPCTTTRNTVQCCEGTFREEDSPENCRKRTGCPGRMVKVGDCQTPWSDIECVH  
181 KESGIIIGVTVAAVLIVAVEVCKSLMKKVLPLYLKICSGGGGDPERVDRSSQRPGEAD  
241 NVLNEIVSILQPTQVPEQEMEVEQPAEPTGVNMLSPGESEHLLLEPAEAERSQRRLLVPA  
301 NEGDPTEITLRQCFFDADFADLVFPDSWEPLMRKLGIMDNEIKVAKABAAGHRDITLYTMLIKW  
361 VNKTGRDASVHTLLDALETGLERAKQKIEDHLLSSGKFMYLEGNADSALS

Fig. 2 B

Apo2	EADLVPEPDSWEP	LMRKKGHMDNEIKVAKAEAA	--CHRDTH
DR4	PANIVPEPDSWDQ	LMHQEDHTKNEDIVVRACTA	--GPGDAI
Apo3/DR3	VMDAVPARRWKEF	VRTLGHRRAETIEAVEVEIGR	--FRDQQ
TNFR1	VVENVPPLRWKEF	VRRRLGLSDHEIDRLLELQNGR	--CLREAO
Fas/Apo1	IRGVMTLSQVKGF	VKKNGVNEAKIDEIKNDNVQD	TAEQKV
Apo2	VTMLIKWVNKTGRD	-ASVHTLDDALETLGEEELAKQKIED	
DR4	QAMLMKKWVNKTGRN	-ASIHTELDALERMEEHAKKEXIQD	
Apo3/DR3	VEMLKRRNRQQQP	--AGLGAVYAAALERMGLDGC	CVEDLRS
TNFR1	VSMLATNRRRTTP	RRRRTAATLELGRVLRDMDL	LGCLCEDIEE
Fas/Apo1	-QILLRNWHQLH	GKKKAY-DTLLIKDDLKKANL	CTLAEXIQT

Fig. 3

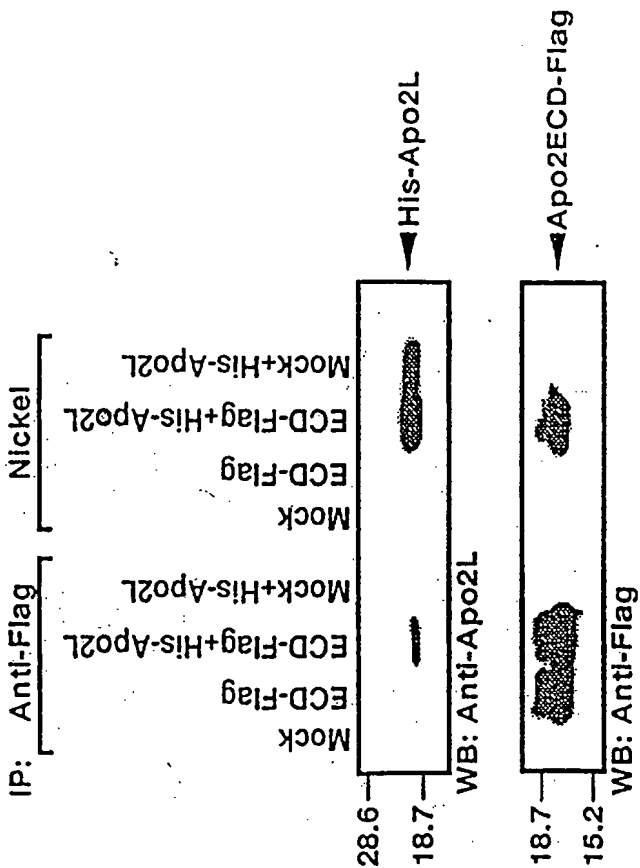
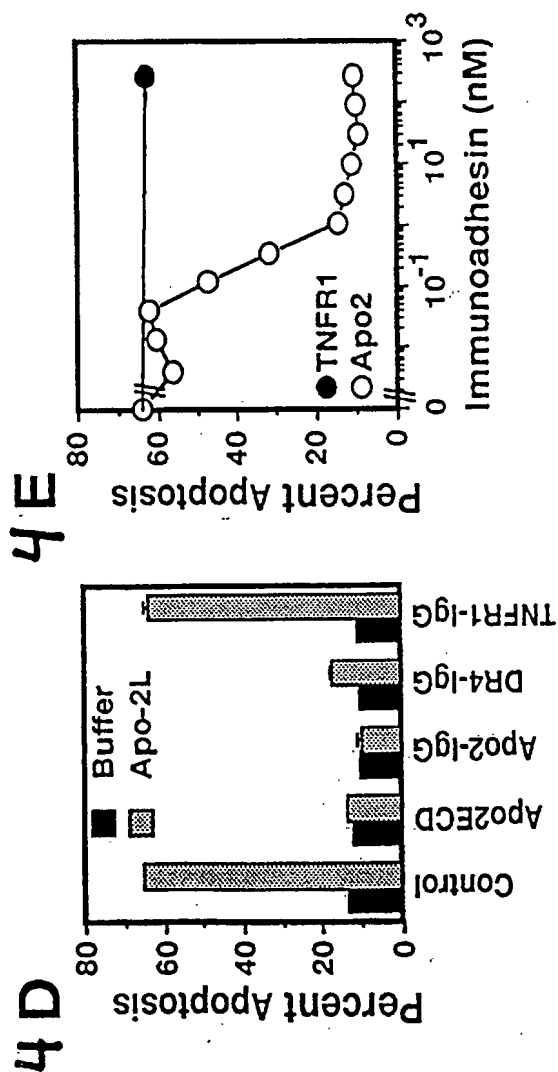
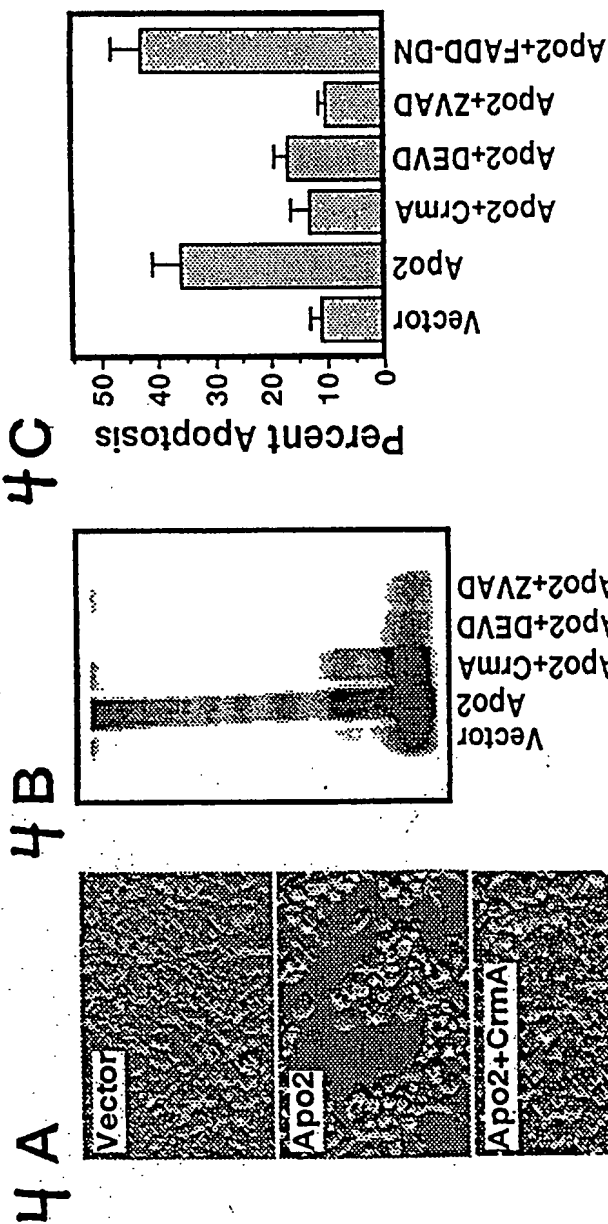


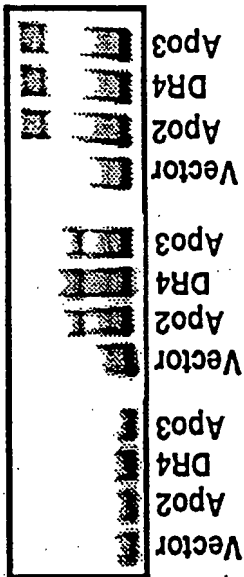
Fig. 4



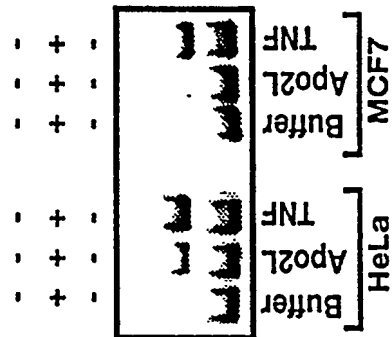


5 A

Unlabelled probe  
Labelled probe  
Anti-p65



5 B



5 C

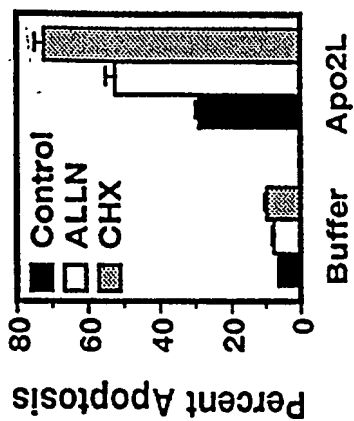


Fig. 5



866020" 5TF42009

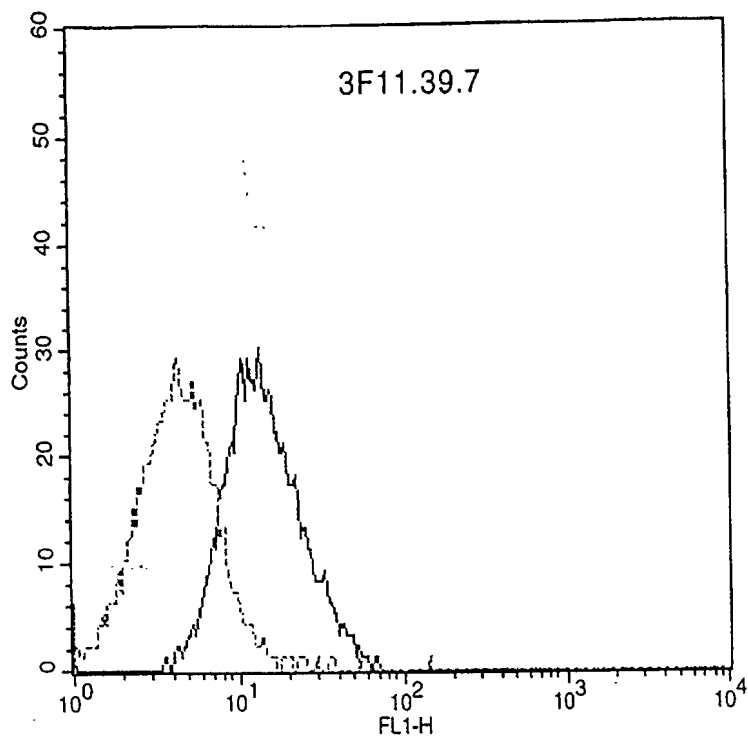


Fig. 7

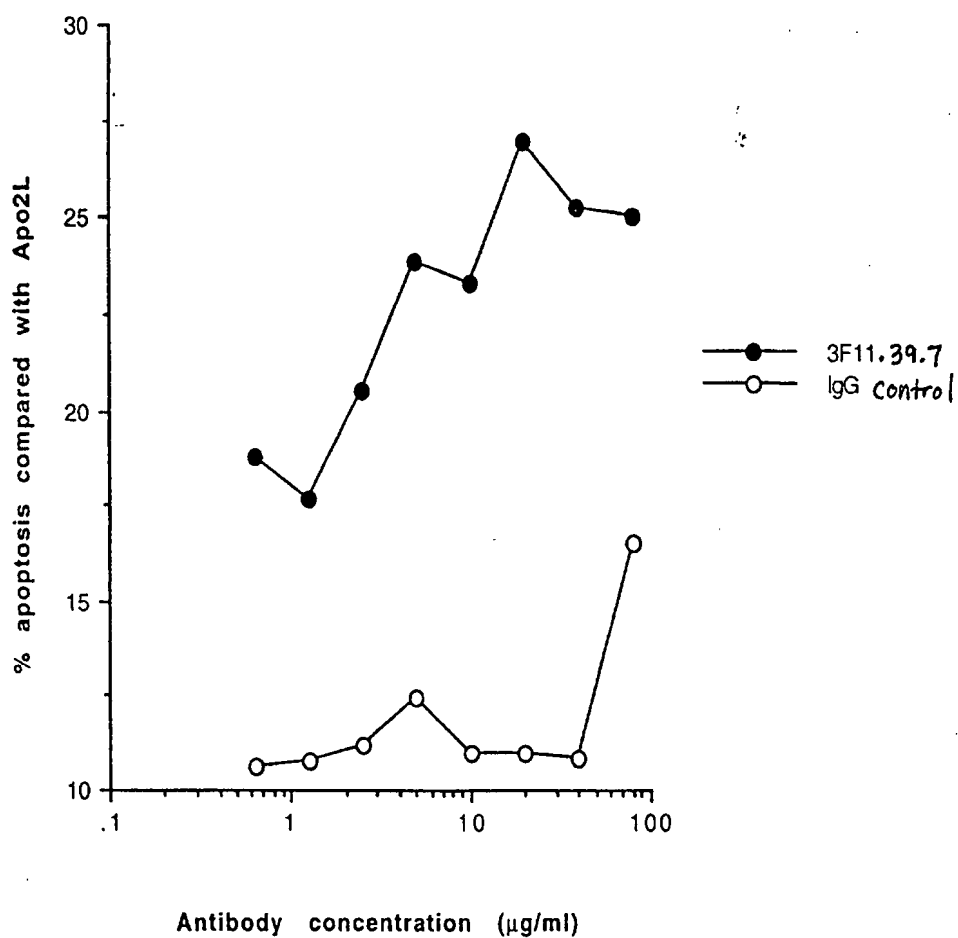


Fig. 8

865020" 674205

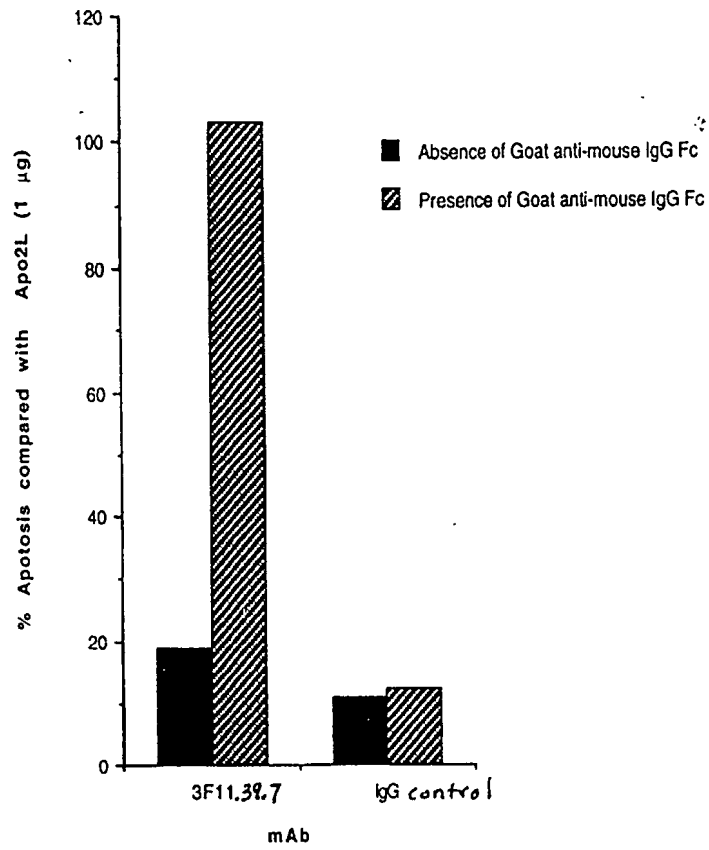


Fig. 9

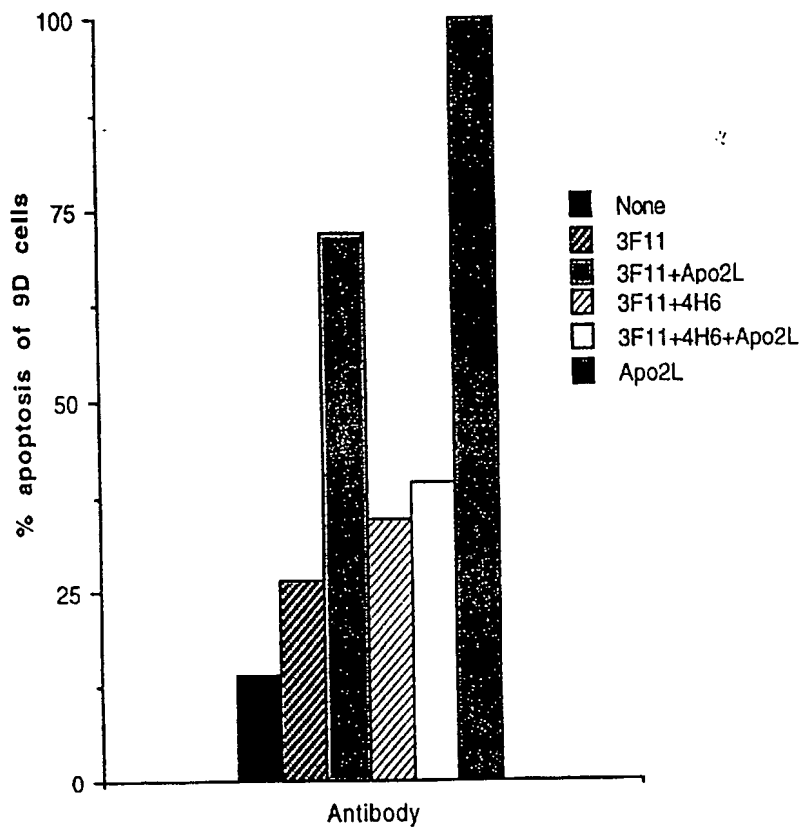


Fig. 10

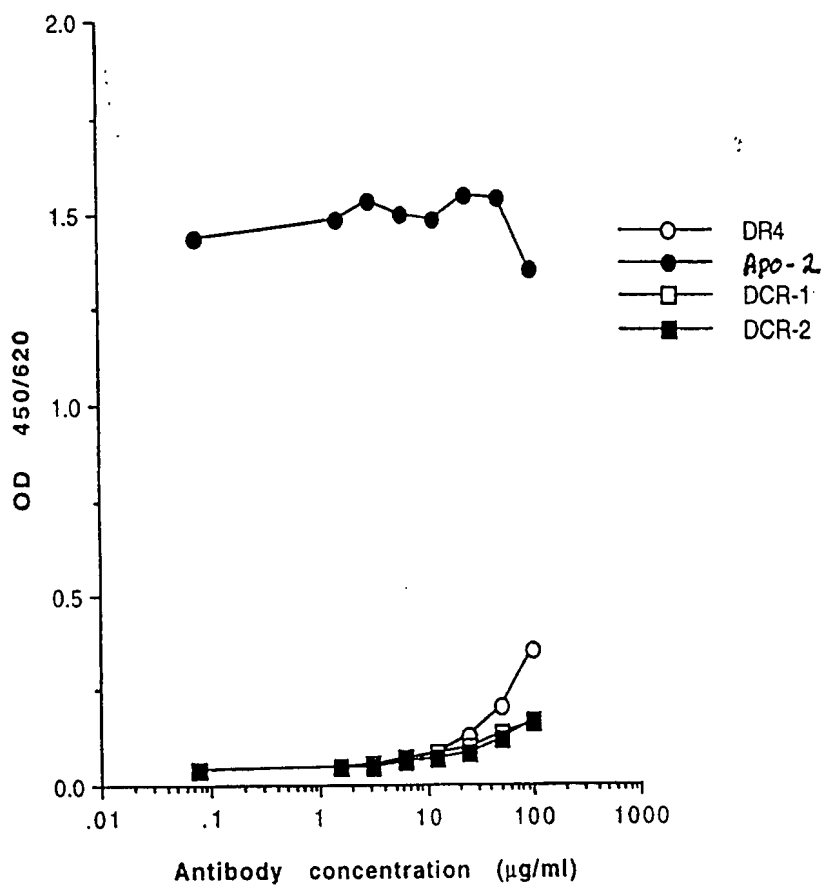


Fig. 11

5

Apo-2 ReceptorRELATED APPLICATIONS

10           This application is a non-provisional application claiming priority under Section 119(e) to provisional application number 60/046,615 filed May 15, 1997 and provisional application number 60/074,119 filed February 9, 1998, the contents of which are hereby incorporated by reference.

15

FIELD OF THE INVENTION

          The present invention relates generally to the identification, isolation, and recombinant production of novel polypeptides, designated herein as Apo-2, and to anti-Apo-2  
20   antibodies.

BACKGROUND OF THE INVENTIONApoptosis or "Programmed Cell Death"

          Control of cell numbers in mammals is believed to be  
25   determined, in part, by a balance between cell proliferation and cell death. One form of cell death, sometimes referred to as necrotic cell death, is typically characterized as a pathologic form of cell death resulting from some trauma or cellular injury. In contrast, there is another, "physiologic" form of cell death  
30   which usually proceeds in an orderly or controlled manner. This orderly or controlled form of cell death is often referred to as "apoptosis" [see, e.g., Barr et al., Bio/Technology, 12:487-493 (1994); Steller et al., Science, 267:1445-1449 (1995)]. Apoptotic cell death naturally occurs in many physiological processes,  
35   including embryonic development and clonal selection in the immune system [Itoh et al., Cell, 66:233-243 (1991)]. Decreased levels of apoptotic cell death have been associated with a variety of pathological conditions, including cancer, lupus, and herpes virus infection [Thompson, Science, 267:1456-1462 (1995)]. Increased



levels of apoptotic cell death may be associated with a variety of other pathological conditions, including AIDS, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, multiple sclerosis, retinitis pigmentosa, cerebellar degeneration, aplastic anemia, myocardial infarction, stroke, reperfusion injury, and toxin-induced liver disease [see, Thompson, supra].

Apoptotic cell death is typically accompanied by one or more characteristic morphological and biochemical changes in cells, such as condensation of cytoplasm, loss of plasma membrane microvilli, segmentation of the nucleus, degradation of chromosomal DNA or loss of mitochondrial function. A variety of extrinsic and intrinsic signals are believed to trigger or induce such morphological and biochemical cellular changes [Raff, Nature, 356:397-400 (1992); Steller, supra; Sachs et al., Blood, 82:15 (1993)]. For instance, they can be triggered by hormonal stimuli, such as glucocorticoid hormones for immature thymocytes, as well as withdrawal of certain growth factors [Watanabe-Fukunaga et al., Nature, 356:314-317 (1992)]. Also, some identified oncogenes such as *myc*, *rel*, and *E1A*, and tumor suppressors, like *p53*, have been reported to have a role in inducing apoptosis. Certain chemotherapy drugs and some forms of radiation have likewise been observed to have apoptosis-inducing activity [Thompson, supra].

#### TNF Family of Cytokines

Various molecules, such as tumor necrosis factor- $\alpha$  ("TNF- $\alpha$ "), tumor necrosis factor- $\beta$  ("TNF- $\beta$ " or "lymphotoxin"), CD30 ligand, CD27 ligand, CD40 ligand, OX-40 ligand, 4-1BB ligand, Apo-1 ligand (also referred to as Fas ligand or CD95 ligand), and Apo-2 ligand (also referred to as TRAIL) have been identified as members of the tumor necrosis factor ("TNF") family of cytokines [See, e.g., Gruss and Dower, Blood, 85:3378-3404 (1995); Wiley et al., Immunity, 3:673-682 (1995); Pitti et al., J. Biol. Chem., 271:12687-12690 (1996); WO 97/01633 published January 16, 1997]. Among these molecules, TNF- $\alpha$ , TNF- $\beta$ , CD30 ligand, 4-1BB ligand, Apo-1 ligand, and Apo-2 ligand (TRAIL) have been reported to be involved in apoptotic cell death. Both TNF- $\alpha$  and TNF- $\beta$  have been reported to induce apoptotic death in susceptible tumor cells [Schmid et al., Proc. Natl. Acad. Sci., 83:1881 (1986); Dealtry et

al., Eur. J. Immunol., 17:689 (1987)]. Zheng et al. have reported that TNF- $\alpha$  is involved in post-stimulation apoptosis of CD8-positive T cells [Zheng et al., Nature, 377:348-351 (1995)]. Other investigators have reported that CD30 ligand may be involved in deletion of self-reactive T cells in the thymus [Amakawa et al., Cold Spring Harbor Laboratory Symposium on Programmed Cell Death, Abstr. No. 10, (1995)].

Mutations in the mouse Fas/Apo-1 receptor or ligand genes (called *lpr* and *gld*, respectively) have been associated with some autoimmune disorders, indicating that Apo-1 ligand may play a role in regulating the clonal deletion of self-reactive lymphocytes in the periphery [Krammer et al., Curr. Op. Immunol., 6:279-289 (1994); Nagata et al., Science, 267:1449-1456 (1995)]. Apo-1 ligand is also reported to induce post-stimulation apoptosis in CD4-positive T lymphocytes and in B lymphocytes, and may be involved in the elimination of activated lymphocytes when their function is no longer needed [Krammer et al., supra; Nagata et al., supra]. Agonist mouse monoclonal antibodies specifically binding to the Apo-1 receptor have been reported to exhibit cell killing activity that is comparable to or similar to that of TNF- $\alpha$  [Yonehara et al., J. Exp. Med., 169:1747-1756 (1989)].

#### TNF Family of Receptors

Induction of various cellular responses mediated by such TNF family cytokines is believed to be initiated by their binding to specific cell receptors. Two distinct TNF receptors of approximately 55-kDa (TNFR1) and 75-kDa (TNFR2) have been identified [Hohman et al., J. Biol. Chem., 264:14927-14934 (1989); Brockhaus et al., Proc. Natl. Acad. Sci., 87:3127-3131 (1990); EP 417,563, published March 20, 1991] and human and mouse cDNAs corresponding to both receptor types have been isolated and characterized [Loetscher et al., Cell, 61:351 (1990); Schall et al., Cell, 61:361 (1990); Smith et al., Science, 248:1019-1023 (1990); Lewis et al., Proc. Natl. Acad. Sci., 88:2830-2834 (1991); Goodwin et al., Mol. Cell. Biol., 11:3020-3026 (1991)]. Extensive polymorphisms have been associated with both TNF receptor genes [see, e.g., Takao et al., Immunogenetics, 37:199-203 (1993)]. Both TNFRs share the typical structure of cell surface receptors

including extracellular, transmembrane and intracellular regions. The extracellular portions of both receptors are found naturally also as soluble TNF-binding proteins [Nophar, Y. et al., EMBO J., 9:3269 (1990); and Kohno, T. et al., Proc. Natl. Acad. Sci. U.S.A., 87:8331 (1990)]. The cloning of recombinant soluble TNF receptors was reported by Hale et al. [J. Cell. Biochem. Supplement 15F, 1991, p. 113 (P424)].

The extracellular portion of type 1 and type 2 TNFRs (TNFR1 and TNFR2) contains a repetitive amino acid sequence pattern of four cysteine-rich domains (CRDs) designated 1 through 4, starting from the NH<sub>2</sub>-terminus. Each CRD is about 40 amino acids long and contains 4 to 6 cysteine residues at positions which are well conserved [Schall et al., supra; Loetscher et al., supra; Smith et al., supra; Nophar et al., supra; Kohno et al., supra]. In TNFR1, the approximate boundaries of the four CRDs are as follows: CRD1- amino acids 14 to about 53; CRD2- amino acids from about 54 to about 97; CRD3- amino acids from about 98 to about 138; CRD4- amino acids from about 139 to about 167. In TNFR2, CRD1 includes amino acids 17 to about 54; CRD2- amino acids from about 55 to about 97; CRD3- amino acids from about 98 to about 140; and CRD4- amino acids from about 141 to about 179 [Banner et al., Cell, 73:431-435 (1993)]. The potential role of the CRDs in ligand binding is also described by Banner et al., supra.

A similar repetitive pattern of CRDs exists in several other cell-surface proteins, including the p75 nerve growth factor receptor (NGFR) [Johnson et al., Cell, 47:545 (1986); Radeke et al., Nature, 325:593 (1987)], the B cell antigen CD40 [Stamenkovic et al., EMBO J., 8:1403 (1989)], the T cell antigen OX40 [Mallet et al., EMBO J., 9:1063 (1990)] and the Fas antigen [Yonehara et al., supra and Itoh et al., supra]. CRDs are also found in the soluble TNFR (sTNFR)-like T2 proteins of the Shope and myxoma poxviruses [Upton et al., Virology, 160:20-29 (1987); Smith et al., Biochem. Biophys. Res. Commun., 176:335 (1991); Upton et al., Virology, 184:370 (1991)]. Optimal alignment of these sequences indicates that the positions of the cysteine residues are well conserved. These receptors are sometimes collectively referred to as members of the TNF/NGF receptor superfamily. Recent studies on p75NGFR showed that the deletion of CRD1 [Welcher, A.A. et al., Proc. Natl. Acad. Sci. USA, 88:159-163 (1991)] or a 5-amino acid insertion in

this domain [Yan, H. and Chao, M.V., J. Biol. Chem., 266:12099-12104 (1991)] had little or no effect on NGF binding [Yan, H. and Chao, M.V., supra]. p75 NGFR contains a proline-rich stretch of about 60 amino acids, between its CRD4 and transmembrane region, which is not involved in NGF binding [Peetre, C. et al., Eur. J. Hematol., 41:414-419 (1988); Seckinger, P. et al., J. Biol. Chem., 264:11966-11973 (1989); Yan, H. and Chao, M.V., supra]. A similar proline-rich region is found in TNFR2 but not in TNFR1.

Itoh et al. disclose that the Apo-1 receptor can signal an apoptotic cell death similar to that signaled by the 55-kDa TNFR1 [Itoh et al., supra]. Expression of the Apo-1 antigen has also been reported to be down-regulated along with that of TNFR1 when cells are treated with either TNF- $\alpha$  or anti-Apo-1 mouse monoclonal antibody [Krammer et al., supra; Nagata et al., supra]. Accordingly, some investigators have hypothesized that cell lines that co-express both Apo-1 and TNFR1 receptors may mediate cell killing through common signaling pathways [Id.].

The TNF family ligands identified to date, with the exception of lymphotoxin- $\alpha$ , are type II transmembrane proteins, whose C-terminus is extracellular. In contrast, the receptors in the TNF receptor (TNFR) family identified to date are type I transmembrane proteins. In both the TNF ligand and receptor families, however, homology identified between family members has been found mainly in the extracellular domain ("ECD"). Several of the TNF family cytokines, including TNF- $\alpha$ , Apo-1 ligand and CD40 ligand, are cleaved proteolytically at the cell surface; the resulting protein in each case typically forms a homotrimeric molecule that functions as a soluble cytokine. TNF receptor family proteins are also usually cleaved proteolytically to release soluble receptor ECDs that can function as inhibitors of the cognate cytokines.

Recently, other members of the mammalian TNFR family have been identified. In Marsters et al., Curr. Biol., 6:750 (1996), investigators describe a full length native sequence human polypeptide, called Apo-3, which exhibits similarity to the TNFR family in its extracellular cysteine-rich repeats and resembles TNFR1 and CD95 in that it contains a cytoplasmic death domain sequence [see also Marsters et al., Curr. Biol., 6:1669 (1996)].

Apo-3 has also been referred to by other investigators as DR3, wsl-1 and TRAMP [Chinnaiyan et al., Science, 274:990 (1996); Kitson et al., Nature, 384:372 (1996); Bodmer et al., Immunity, 6:79 (1997)].

Pan et al. have disclosed another TNF receptor family member referred to as "DR4" [Pan et al., Science, 276:111-113 (1997)]. The DR4 was reported to contain a cytoplasmic death domain capable of engaging the cell suicide apparatus. Pan et al. disclose that DR4 is believed to be a receptor for the ligand known as Apo-2 ligand or TRAIL.

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#### The Apoptosis-Inducing Signaling Complex

As presently understood, the cell death program contains at least three important elements - activators, inhibitors, and effectors; in *C. elegans*, these elements are encoded respectively by three genes, *Ced-4*, *Ced-9* and *Ced-3* [Steller, Science, 267:1445 (1995); Chinnaiyan et al., Science, 275:1122-1126 (1997)]. Two of the TNFR family members, TNFR1 and Fas/Apo1 (CD95), can activate apoptotic cell death [Chinnaiyan and Dixit, Current Biology, 6:555-562 (1996); Fraser and Evan, Cell, 85:781-784 (1996)]. TNFR1 is also known to mediate activation of the transcription factor, NF- $\kappa$ B [Tartaglia et al., Cell, 74:845-853 (1993); Hsu et al., Cell, 84:299-308 (1996)]. In addition to some ECD homology, these two receptors share homology in their intracellular domain (ICD) in an oligomerization interface known as the death domain [Tartaglia et al., supra; Nagata, Cell, 88:355 (1997)]. Death domains are also found in several metazoan proteins that regulate apoptosis, namely, the *Drosophila* protein, Reaper, and the mammalian proteins referred to as FADD/MORT1, TRADD, and RIP [Cleaveland and Ihle, Cell, 81:479-482 (1995)]. Using the yeast-two hybrid system, Raven et al. report the identification of protein, wsl-1, which binds to the TNFR1 death domain [Raven et al., Programmed Cell Death Meeting, September 20-24, 1995, Abstract at page 127; Raven et al., European Cytokine Network, 7:Abstr. 82 at page 210 (April-June 1996)]. The wsl-1 protein is described as being homologous to TNFR1 (48% identity) and having a restricted tissue distribution. According to Raven et al., the tissue distribution of wsl-1 is significantly different from the TNFR1 binding protein, TRADD.

Upon ligand binding and receptor clustering, TNFR1 and CD95 are believed to recruit FADD into a death-inducing signalling

complex. CD95 purportedly binds FADD directly, while TNFR1 binds FADD indirectly via TRADD [Chinnaiyan et al., Cell, 81:505-512 (1995); Boldin et al., J. Biol. Chem., 270:387-391 (1995); Hsu et al., supra; Chinnaiyan et al., J. Biol. Chem., 271:4961-4965 (1996)]. It has been reported that FADD serves as an adaptor protein which recruits the Ced-3-related protease, MACH $\alpha$ /FLICE (caspase 8), into the death signalling complex [Boldin et al., Cell, 85:803-815 (1996); Muzio et al., Cell, 85:817-827 (1996)]. MACH $\alpha$ /FLICE appears to be the trigger that sets off a cascade of apoptotic proteases, including the interleukin-1 $\beta$  converting enzyme (ICE) and CPP32/Yama, which may execute some critical aspects of the cell death programme [Fraser and Evan, supra].

It was recently disclosed that programmed cell death involves the activity of members of a family of cysteine proteases related to the *C. elegans* cell death gene, *ced-3*, and to the mammalian IL-1-converting enzyme, ICE. The activity of the ICE and CPP32/Yama proteases can be inhibited by the product of the cowpox virus gene, *crmA* [Ray et al., Cell, 69:597-604 (1992); Tewari et al., Cell, 81:801-809 (1995)]. Recent studies show that CrmA can inhibit TNFR1- and CD95-induced cell death [Enari et al., Nature, 375:78-81 (1995); Tewari et al., J. Biol. Chem., 270:3255-3260 (1995)].

As reviewed recently by Tewari et al., TNFR1, TNFR2 and CD40 modulate the expression of proinflammatory and costimulatory cytokines, cytokine receptors, and cell adhesion molecules through activation of the transcription factor, NF- $\kappa$ B [Tewari et al., Curr. Op. Genet. Develop., 6:39-44 (1996)]. NF- $\kappa$ B is the prototype of a family of dimeric transcription factors whose subunits contain conserved Rel regions [Verma et al., Genes Develop., 9:2723-2735 (1996); Baldwin, Ann. Rev. Immunol., 14:649-681 (1996)]. In its latent form, NF- $\kappa$ B is complexed with members of the I $\kappa$ B inhibitor family; upon inactivation of the I $\kappa$ B in response to certain stimuli, released NF- $\kappa$ B translocates to the nucleus where it binds to specific DNA sequences and activates gene transcription.

For a review of the TNF family of cytokines and their receptors, see Gruss and Dower, supra.

SUMMARY OF THE INVENTION

Applicants have identified cDNA clones that encode novel polypeptides, designated in the present application as "Apo-2." It is believed that Apo-2 is a member of the TNFR family; full-length native sequence human Apo-2 polypeptide exhibits some similarities to some known TNFRs, including a cytoplasmic death domain region. Full-length native sequence human Apo-2 also exhibits similarity to the TNFR family in its extracellular cysteine-rich repeats. Apo-2 polypeptide has been found to be capable of triggering caspase-dependent apoptosis and activating NF- $\kappa$ B. Applicants surprisingly found that a soluble extracellular domain of Apo-2 binds Apo-2 ligand ("Apo-2L") and can inhibit Apo-2 ligand function. It is presently believed that Apo-2 ligand can signal via at least two different receptors, DR4 and the newly described Apo-2 herein.

In one embodiment, the invention provides isolated Apo-2 polypeptide. In particular, the invention provides isolated native sequence Apo-2 polypeptide, which in one embodiment, includes an amino acid sequence comprising residues 1 to 411 of Figure 1 (SEQ ID NO:1). In other embodiments, the isolated Apo-2 polypeptide comprises at least about 80% amino acid sequence identity with native sequence Apo-2 polypeptide comprising residues 1 to 411 of Figure 1 (SEQ ID NO:1). Optionally, the Apo-2 polypeptide is obtained or obtainable by expressing the polypeptide encoded by the cDNA insert of the vector deposited as ATCC 209021.

In another embodiment, the invention provides an isolated extracellular domain (ECD) sequence of Apo-2. Optionally, the isolated extracellular domain sequence comprises amino acid residues 54 to 182 of Fig. 1 (SEQ ID NO:1).

In another embodiment, the invention provides an isolated death domain sequence of Apo-2. Optionally, the isolated death domain sequence comprises amino acid residues 324 to 391 of Fig. 1 (SEQ ID NO:1).

In another embodiment, the invention provides chimeric molecules comprising Apo-2 polypeptide fused to a heterologous polypeptide or amino acid sequence. An example of such a chimeric molecule comprises an Apo-2 fused to an immunoglobulin sequence. Another example comprises an extracellular domain sequence of Apo-2 fused to a heterologous polypeptide or amino acid sequence, such as an immunoglobulin sequence.

In another embodiment, the invention provides an isolated nucleic acid molecule encoding Apo-2 polypeptide. In one aspect, the nucleic acid molecule is RNA or DNA that encodes an Apo-2 polypeptide or a particular domain of Apo-2, or is  
5 complementary to such encoding nucleic acid sequence, and remains stably bound to it under at least moderate, and optionally, under high stringency conditions. Such complementary nucleic acid may be fully complementary to the entire length of the RNA or DNA. It is contemplated that the complementary nucleic acid may also be  
10 complementary to only a fragment of the RNA or DNA nucleotide sequence. In one embodiment, the nucleic acid sequence is selected from:

(a) the coding region of the nucleic acid sequence of Figure 1 (SEQ ID NO:2) that codes for residue 1 to residue 411  
15 (i.e., nucleotides 140-142 through 1370-1372), inclusive;

(b) the coding region of the nucleic acid sequence of Figure 1 (SEQ ID NO:2) that codes for residue 1 to residue 182 (i.e., nucleotides 140-142 through 683-685), inclusive;

(c) the coding region of the nucleic acid sequence of  
20 Figure 1 (SEQ ID NO:2) that codes for residue 54 to residue 182 (i.e., nucleotides 299-301 through 683-685), inclusive;

(d) the coding region of the nucleic acid sequence of Figure 1 (SEQ ID NO:2) that codes for residue 324 to residue 391 (i.e., nucleotides 1109-1111 through 1310-1312), inclusive; or

25 (e) a sequence corresponding to the sequence of (a), (b), (c) or (d) within the scope of degeneracy of the genetic code. The isolated nucleic acid may comprise the Apo-2 polypeptide cDNA insert of the vector deposited as ATCC 209021 which includes the nucleotide sequence encoding Apo-2 polypeptide.

30 In a further embodiment, the invention provides a vector comprising the nucleic acid molecule encoding the Apo-2 polypeptide or particular domain of Apo-2. A host cell comprising the vector or the nucleic acid molecule is also provided. A method of producing Apo-2 is further provided.

35 In another embodiment, the invention provides an antibody which specifically binds to Apo-2. The antibody may be an agonistic, antagonistic or neutralizing antibody. Single-chain antibodies and dimeric molecules, in particular homodimeric molecules, comprising Apo-2 antibody are also provided.



In another embodiment, the invention provides non-human, transgenic or knock-out animals.

A further embodiment of the invention provides articles of manufacture and kits that include Apo-2 or Apo-2 antibodies.

5

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the nucleotide sequence of a native sequence human Apo-2 cDNA (SEQ ID NO:2) and its derived amino acid sequence (SEQ ID NO:1).

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Figure 2A shows the derived amino acid sequence of a native sequence human Apo-2 - the putative signal sequence is underlined, the putative transmembrane domain is boxed, and the putative death domain sequence is dash underlined. The cysteines of the two cysteine-rich domains are individually underlined.

15

Figure 2B shows an alignment and comparison of the death domain sequences of native sequence human Apo-2, DR4, Apo-3/DR3, TNFR1, and Fas/Apo-1 (CD95). Asterisks indicate residues that are essential for death signaling by TNFR1 [Tartaglia et al., *supra*].

Figure 3 shows the interaction of the Apo-2 ECD with Apo-2L. Supernatants from mock-transfected 293 cells or from 293 cells transfected with Flag epitope-tagged Apo-2 ECD were incubated with poly-His-tagged Apo-2L and subjected to immunoprecipitation with anti-Flag conjugated or Nickel conjugated agarose beads. The precipitated proteins were resolved by electrophoresis on polyacrylamide gels, and detected by immunoblot with anti-Apo-2L or anti-Flag antibody.

20

Figure 4 shows the induction of apoptosis by Apo-2 and inhibition of Apo-2L activity by soluble Apo-2 ECD. Human 293 cells (A, B) or HeLa cells (C) were transfected by pRK5 vector or by pRK5-based plasmids encoding Apo-2 and/or CrmA. Apoptosis was assessed by morphology (A), DNA fragmentation (B), or by FACS (C-E). Soluble Apo-2L was pre-incubated with buffer or affinity-purified Apo-2 ECD together with anti-Flag antibody or Apo-2 ECD immunoadhesin or DR4 or TNFR1 immunoadhesins and added to HeLa cells. The cells were later analyzed for apoptosis (D). Dose-response analysis using Apo-2L with Apo-2 ECD immunoadhesin was also determined (E).

25

Figure 5 shows activation of NF-KB by Apo-2, DR4, and Apo-2L. (A) HeLa cells were transfected with expression plasmids

encoding the indicated proteins. Nuclear extracts were prepared and analyzed by an electrophoretic mobility shift assay. (B) HeLa cells or MCF7 cells were treated with buffer, Apo-2L or TNF-alpha and assayed for NF-kB activity. (C) HeLa cells were preincubated  
5 with buffer, ALLN or cyclohexamide before addition of Apo-2L. Apoptosis was later analyzed by FACS.

Figure 6A shows expression of Apo-2 mRNA in human tissues as analyzed by Northern hybridization of human tissue poly A RNA blots.

10 Figure 6B shows expression of Apo-2 mRNA in human cancer cell lines as analyzed by Northern hybridization of human cancer cell line poly A RNA blots.

Figure 7 shows the FACS analysis of an Apo-2 antibody, 3F11.39.7 (illustrated by the bold lines) as compared to IgG  
15 controls (dotted lines). The 3F11.39.7 antibody recognized the Apo-2 receptor expressed in human 9D cells.

Figure 8 is a graph showing percent (%) apoptosis induced in 9D cells by Apo-2 antibody 3F11.39.7, in the absence of goat anti-mouse IgG Fc.

20 Figure 9 is a bar diagram showing percent (%) apoptosis, as compared to Apo-2L, in 9D cells by Apo-2 antibody 3F11.39.7 in the presence or absence of goat anti-mouse IgG Fc.

Figure 10 is a bar diagram illustrating the ability of Apo-2 antibody 3F11.39.7 to block the apoptosis induced by Apo-2L  
25 in 9D cells.

Figure 11 is a graph showing results of an ELISA testing binding of Apo-2 antibody 3F11.39.7 to Apo-2 and to other known Apo-2L receptors referred to as DR4, DcR1, and DcR2.

Figure 12A is a graph showing the results of an ELISA  
30 assay evaluating binding of the 16E2 antibody to Apo-2, DR4, DcR1, DcR2 and CD4-Ig.

Figure 12B is a graph showing the results of an ELISA assay evaluating binding of the 20E6 antibody to Apo-2, DR4, DcR1, DcR2 and CD4-Ig.

35 Figure 12C is a graph showing the results of an ELISA assay evaluating binding of the 24C4 antibody to Apo-2, DR4, DcR1, DcR2 and CD4-Ig.

Figure 13A is a graph showing agonistic activity of the 16E2 antibody, as compared to Apo-2L, in an apoptosis assay (crystal violet stain) using SK-MES-1 cells.

Figure 13B is a bar diagram showing agonistic activity of the 16E2 antibody, as compared to 7D5 scFv antibody (an anti-tissue factor antibody), in an apoptosis assay (crystal violet stain) using SK-MES-1 cells.

Figure 13C is a bar diagram showing agonistic activity of the 16E2 antibody, as compared to 7D5 scFv antibody, in an apoptosis assay (annexin V-biotin/streptavidin-[S<sup>35</sup>]) using SK-MES-1 cells.

Figure 14A is a graph showing agonistic activity of the 20E6 antibody, as compared to Apo-2L, in an apoptosis assay (crystal violet stain) using SK-MES-1 cells.

Figure 14B is a graph showing agonistic activity of the 20E6 antibody by a comparison between results obtained in the crystal violet and annexin V-biotin/streptavidin-[S<sup>35</sup>] apoptosis assays.

Figure 14C is a graph showing agonistic activity of gD-tagged 16E2 antibody, as compared to Apo-2L, in an apoptosis assay (crystal violet stain) using SK-MES-1 cells.

Figure 15A shows the nucleotide sequence of the single chain antibody (scFv) fragment referred to as 16E2 (SEQ ID NO:6).

Figure 15B shows the nucleotide sequence of the single chain antibody (scFv) fragment referred to as 20E6 (SEQ ID NO:7).

Figure 15C shows the nucleotide sequence of the single chain antibody (scFv) fragment referred to as 24C4 (SEQ ID NO:8).

Figure 16 shows the single chain antibody (scFv) fragments referred to as 16E2, 20E6 and 24C4, with the respective amino acid sequences for the signal sequence and the heavy and light chain CDR regions identified (CDR1, CDR2, and CDR3 regions are underlined).

## DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

### I. Definitions

The terms "Apo-2 polypeptide" and "Apo-2" when used herein encompass native sequence Apo-2 and Apo-2 variants (which

are further defined herein). These terms encompass Apo-2 from a variety of mammals, including humans. The Apo-2 may be isolated from a variety of sources, such as from human tissue types or from another source, or prepared by recombinant or synthetic methods.

5 A "native sequence Apo-2" comprises a polypeptide having the same amino acid sequence as an Apo-2 derived from nature. Thus, a native sequence Apo-2 can have the amino acid sequence of naturally-occurring Apo-2 from any mammal. Such native sequence Apo-2 can be isolated from nature or can be produced by recombinant  
10 or synthetic means. The term "native sequence Apo-2" specifically encompasses naturally-occurring truncated or secreted forms of the Apo-2 (e.g., an extracellular domain sequence), naturally-occurring variant forms (e.g., alternatively spliced forms) and naturally-occurring allelic variants of the Apo-2. A naturally-occurring  
15 variant form of the Apo-2 includes an Apo-2 having an amino acid substitution at residue 410 in the amino acid sequence shown in Figure 1 (SEQ ID NO:1). In one embodiment of such naturally-occurring variant form, the leucine residue at position 410 is substituted by a methionine residue. In Fig. 1 (SEQ ID NO:1), the  
20 amino acid residue at position 410 is identified as "Xaa" to indicate that the amino acid may, optionally, be either leucine or methionine. In Fig. 1 (SEQ ID NO:2), the nucleotide at position 1367 is identified as "W" to indicate that the nucleotide may be either adenine (A) or thymine (T) or uracil (U). In one embodiment  
25 of the invention, the native sequence Apo-2 is a mature or full-length native sequence Apo-2 comprising amino acids 1 to 411 of Fig. 1 (SEQ ID NO:1). Optionally, the Apo-2 is obtained or obtainable by expressing the polypeptide encoded by the cDNA insert of the vector deposited as ATCC 209021.

30 The "Apo-2 extracellular domain" or "Apo-2 ECD" refers to a form of Apo-2 which is essentially free of the transmembrane and cytoplasmic domains of Apo-2. Ordinarily, Apo-2 ECD will have less than 1% of such transmembrane and/or cytoplasmic domains and preferably, will have less than 0.5% of such domains. Optionally,  
35 Apo-2 ECD will comprise amino acid residues 54 to 182 of Fig. 1 (SEQ ID NO:1) or amino acid residues 1 to 182 of Fig. 1 (SEQ ID NO:1). Optionally, Apo-2 ECD will comprise one or more cysteine-rich domains, and preferably, one or both of the cysteine-rich domains identified herein (see Figure 2A). It will be understood

by the skilled artisan that the transmembrane domain identified for the Apo-2 polypeptide herein is identified pursuant to criteria routinely employed in the art for identifying that type of hydrophobic domain. The exact boundaries of a transmembrane domain may vary but most likely by no more than about 5 amino acids at either end of the domain specifically mentioned herein.

"Apo-2 variant" means a biologically active Apo-2 as defined below having at least about 80% amino acid sequence identity with the Apo-2 having the deduced amino acid sequence shown in Fig. 1 (SEQ ID NO:1) for a full-length native sequence human Apo-2 or the sequences identified herein for Apo-2 ECD or death domain. Such Apo-2 variants include, for instance, Apo-2 polypeptides wherein one or more amino acid residues are added, or deleted, at the N- or C-terminus of the sequence of Fig. 1 (SEQ ID NO:1) or the sequences identified herein for Apo-2 ECD or death domain. Ordinarily, an Apo-2 variant will have at least about 80% amino acid sequence identity, more preferably at least about 90% amino acid sequence identity, and even more preferably at least about 95% amino acid sequence identity with the amino acid sequence of Fig. 1 (SEQ ID NO:1) or the sequences identified herein for Apo-2 ECD or death domain.

"Percent (%) amino acid sequence identity" with respect to the Apo-2 sequences identified herein is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the Apo-2 sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as ALIGN™ or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared.

The term "epitope tagged" when used herein refers to a chimeric polypeptide comprising Apo-2 or Apo-2 antibody, or a domain sequence thereof, fused to a "tag polypeptide". The tag

polypeptide has enough residues to provide an epitope against which an antibody can be made, yet is short enough such that it does not interfere with activity of the Apo-2 or Apo-2 antibody. The tag polypeptide preferably also is fairly unique so that the antibody  
5 does not substantially cross-react with other epitopes. Suitable tag polypeptides generally have at least six amino acid residues and usually between about 8 to about 50 amino acid residues (preferably, between about 10 to about 20 residues).

"Isolated," when used to describe the various  
10 polypeptides disclosed herein, means polypeptide that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would typically interfere with diagnostic or therapeutic uses for the polypeptide, and may include  
15 enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In preferred embodiments, the polypeptide will be purified (1) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (2) to homogeneity by SDS-PAGE under non-  
20 reducing or reducing conditions using Coomassie blue or, preferably, silver stain. Isolated polypeptide includes polypeptide in situ within recombinant cells, since at least one component of the Apo-2 natural environment will not be present. Ordinarily, however, isolated polypeptide will be prepared by at  
25 least one purification step.

An "isolated" Apo-2 nucleic acid molecule is a nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the Apo-2 nucleic acid. An  
30 isolated Apo-2 nucleic acid molecule is other than in the form or setting in which it is found in nature. Isolated Apo-2 nucleic acid molecules therefore are distinguished from the Apo-2 nucleic acid molecule as it exists in natural cells. However, an isolated Apo-2 nucleic acid molecule includes Apo-2 nucleic acid molecules  
35 contained in cells that ordinarily express Apo-2 where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence

in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nuclêic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

The term "antibody" is used in the broadest sense and specifically covers anti-Apo-2 monoclonal antibodies (including agonist, antagonist, and blocking or neutralizing antibodies) and anti-Apo-2 antibody compositions with polyepitopic specificity.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally-occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen.

The monoclonal antibodies herein include hybrid and recombinant antibodies produced by splicing a variable (including hypervariable) domain of an anti-Apo-2 antibody with a constant domain, or a light chain with a heavy chain, or a chain from one

species with a chain from another species, or fusions with heterologous proteins, regardless of species of origin or immunoglobulin class or subclass designation, as well as antibody fragments (e.g., Fab, F(ab')<sub>2</sub>, and Fv), so long as they exhibit the desired biological activity. See, e.g. U.S. Pat. No. 4,816,567 and Mage et al., in Monoclonal Antibody Production Techniques and Applications, pp.79-97 (Marcel Dekker, Inc.: New York, 1987).

Thus, the modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler and Milstein, Nature, 256:495 (1975), or may be made by recombinant DNA methods such as described in U.S. Pat. No. 4,816,567. The "monoclonal antibodies" may also be isolated from phage libraries generated using the techniques described in McCafferty et al., Nature, 348:552-554 (1990), for example.

"Single-chain Fv" or "scFv" antibody fragments comprise the V<sub>H</sub> and V<sub>L</sub> domains of antibody, wherein these domains are present in a single polypeptide chain. Generally, the Fv polypeptide further comprises a polypeptide linker between the V<sub>H</sub> and V<sub>L</sub> domains which enables the scFv to form the desired structure for antigen binding. For a review of scFv see, e.g., Pluckthun, The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenberg and Moore eds. Springer-Verlag, New York, pp. 269-315 (1994). The scFv antibody fragments of the present invention include but are not limited to the 16E2, 20E6 and 24C4 antibodies described in detail below. Within the scope of the scFv antibodies of the invention are scFv antibodies comprising V<sub>H</sub> and V<sub>L</sub> domains that include one or more of the CDR regions identified for the 16E2, 20E6 and 24C4 antibodies.

"Humanized" forms of non-human (e.g. murine) antibodies are specific chimeric immunoglobulins, immunoglobulin chains, or fragments thereof (such as Fv, Fab, Fab', F(ab')<sub>2</sub> or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of



the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat, or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, the humanized antibody may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region or domain (Fc), typically that of a human immunoglobulin.

"Biologically active" and "desired biological activity" for the purposes herein means (1) having the ability to modulate apoptosis (either in an agonistic or stimulating manner or in an antagonistic or blocking manner) in at least one type of mammalian cell *in vivo* or *ex vivo*; (2) having the ability to bind Apo-2 ligand; or (3) having the ability to modulate Apo-2 ligand signaling and Apo-2 ligand activity.

The terms "apoptosis" and "apoptotic activity" are used in a broad sense and refer to the orderly or controlled form of cell death in mammals that is typically accompanied by one or more characteristic cell changes, including condensation of cytoplasm, loss of plasma membrane microvilli, segmentation of the nucleus, degradation of chromosomal DNA or loss of mitochondrial function. This activity can be determined and measured, for instance, by cell viability assays, FACS analysis or DNA electrophoresis, all of which are known in the art.

The terms "treating," "treatment," and "therapy" as used herein refer to curative therapy, prophylactic therapy, and preventative therapy.

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer

include but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, blastoma, gastrointestinal cancer, renal cancer, 5 pancreatic cancer, glioblastoma, neuroblastoma, cervical cancer, ovarian cancer, liver cancer, stomach cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, 10 hepatic carcinoma and various types of head and neck cancer.

The term "mammal" as used herein refers to any mammal classified as a mammal, including humans, cows, horses, dogs and cats. In a preferred embodiment of the invention, the mammal is a human.

15

## II. Compositions and Methods of the Invention

The present invention provides newly identified and isolated Apo-2 polypeptides and Apo-2 antibodies. In particular, Applicants have identified and isolated various human Apo-2 20 polypeptides. The properties and characteristics of some of these Apo-2 polypeptides and anti-Apo-2 antibodies are described in further detail in the Examples below. Based upon the properties and characteristics of the Apo-2 polypeptides disclosed herein, it is Applicants' present belief that Apo-2 is a member of the TNFR 25 family.

A description follows as to how Apo-2, as well as Apo-2 chimeric molecules and anti-Apo-2 antibodies, may be prepared.

### A. Preparation of Apo-2

30 The description below relates primarily to production of Apo-2 by culturing cells transformed or transfected with a vector containing Apo-2 nucleic acid. It is of course, contemplated that alternative methods, which are well known in the art, may be employed to prepare Apo-2.

35

#### 1. Isolation of DNA Encoding Apo-2

The DNA encoding Apo-2 may be obtained from any cDNA library prepared from tissue believed to possess the Apo-2 mRNA and to express it at a detectable level. Accordingly, human Apo-2 DNA can be conveniently obtained from a cDNA library prepared from

human tissues, such as the bacteriophage libraries of human pancreas and kidney cDNA described in Example 1. The Apo-2-encoding gene may also be obtained from a genomic library or by oligonucleotide synthesis.

5 Libraries can be screened with probes (such as antibodies to the Apo-2 or oligonucleotides of at least about 20-80 bases) designed to identify the gene of interest or the protein encoded by it. Screening the cDNA or genomic library with the selected probe may be conducted using standard procedures, such as  
10 described in Sambrook et al., Molecular Cloning: A Laboratory Manual (New York: Cold Spring Harbor Laboratory Press, 1989). An alternative means to isolate the gene encoding Apo-2 is to use PCR methodology [Sambrook et al., supra; Dieffenbach et al., PCR Primer: A Laboratory Manual (Cold Spring Harbor Laboratory Press,  
15 1995)].

A preferred method of screening employs selected oligonucleotide sequences to screen cDNA libraries from various human tissues. Example 1 below describes techniques for screening a cDNA library. The oligonucleotide sequences selected as probes  
20 should be of sufficient length and sufficiently unambiguous that false positives are minimized. The oligonucleotide is preferably labeled such that it can be detected upon hybridization to DNA in the library being screened. Methods of labeling are well known in the art, and include the use of radiolabels like <sup>32</sup>P-labeled ATP,  
25 biotinylation or enzyme labeling. Hybridization conditions, including moderate stringency and high stringency, are provided in Sambrook et al., supra.

Nucleic acid having all the protein coding sequence may be obtained by screening selected cDNA or genomic libraries using  
30 the deduced amino acid sequence disclosed herein for the first time, and, if necessary, using conventional primer extension procedures as described in Sambrook et al., supra, to detect precursors and processing intermediates of mRNA that may not have been reverse-transcribed into cDNA.

35 Apo-2 variants can be prepared by introducing appropriate nucleotide changes into the Apo-2 DNA, or by synthesis of the desired Apo-2 polypeptide. Those skilled in the art will appreciate that amino acid changes may alter post-translational processes of the Apo-2, such as changing the number or position of

glycosylation sites or altering the membrane anchoring characteristics.

Variations in the native full-length sequence Apo-2 or in various domains of the Apo-2 described herein, can be made, for example, using any of the techniques and guidelines for conservative and non-conservative mutations set forth, for instance, in U.S. Pat. No. 5,364,934. Variations may be a substitution, deletion or insertion of one or more codons encoding the Apo-2 that results in a change in the amino acid sequence of the Apo-2 as compared with the native sequence Apo-2. Optionally the variation is by substitution of at least one amino acid with any other amino acid in one or more of the domains of the Apo-2 molecule. The variations can be made using methods known in the art such as oligonucleotide-mediated (site-directed) mutagenesis, alanine scanning, and PCR mutagenesis. Site-directed mutagenesis [Carter et al., Nucl. Acids Res., 13:4331 (1986); Zoller et al., Nucl. Acids Res., 10:6487 (1987)], cassette mutagenesis [Wells et al., Gene, 34:315 (1985)], restriction selection mutagenesis [Wells et al., Philos. Trans. R. Soc. London SerA, 317:415 (1986)] or other known techniques can be performed on the cloned DNA to produce the Apo-2 variant DNA.

Scanning amino acid analysis can also be employed to identify one or more amino acids along a contiguous sequence which are involved in the interaction with a particular ligand or receptor. Among the preferred scanning amino acids are relatively small, neutral amino acids. Such amino acids include alanine, glycine, serine, and cysteine. Alanine is the preferred scanning amino acid among this group because it eliminates the side-chain beyond the beta-carbon and is less likely to alter the main-chain conformation of the variant. Alanine is also preferred because it is the most common amino acid. Further, it is frequently found in both buried and exposed positions [Creighton, The Proteins, (W.H. Freeman & Co., N.Y.); Chothia, J. Mol. Biol., 150:1 (1976)]. If alanine substitution does not yield adequate amounts of variant, an isoteric amino acid can be used.

Once selected Apo-2 variants are produced, they can be contacted with, for instance, Apo-2L, and the interaction, if any, can be determined. The interaction between the Apo-2 variant and Apo-2L can be measured by an *in vitro* assay, such as described in

the Examples below. While any number of analytical measurements can be used to compare activities and properties between a native sequence Apo-2 and an Apo-2 variant, a convenient one for binding is the dissociation constant  $K_d$  of the complex formed between the Apo-2 variant and Apo-2L as compared to the  $K_d$  for the native sequence Apo-2. Generally, a  $\geq 3$ -fold increase or decrease in  $K_d$  per substituted residue indicates that the substituted residue(s) is active in the interaction of the native sequence Apo-2 with the Apo-2L.

Optionally, representative sites in the Apo-2 sequence suitable for mutagenesis would include sites within the extracellular domain, and particularly, within one or both of the cysteine-rich domains. Such variations can be accomplished using the methods described above.

## 2. Insertion of Nucleic Acid into A Replicable Vector

The nucleic acid (e.g., cDNA or genomic DNA) encoding Apo-2 may be inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. Various vectors are publicly available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence, each of which is described below.

### (i) Signal Sequence Component

The Apo-2 may be produced recombinantly not only directly, but also as a fusion polypeptide with a heterologous polypeptide, which may be a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. In general, the signal sequence may be a component of the vector, or it may be a part of the Apo-2 DNA that is inserted into the vector. The heterologous signal sequence selected preferably is one that is recognized and processed (i.e., cleaved by a signal peptidase) by the host cell. The signal sequence may be a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion the signal sequence may be, e.g., the yeast invertase leader, alpha factor leader (including *Saccharomyces* and *Kluyveromyces*  $\alpha$ -factor leaders, the latter described in U.S. Pat. No. 5,010,182), or acid

phosphatase leader, the *C. albicans* glucoamylase leader (EP 362,179 published 4 April 1990), or the signal described in WO 90/13646 published 15 November 1990. In mammalian cell expression the native Apo-2 presequence that normally directs insertion of Apo-2 in the cell membrane of human cells *in vivo* is satisfactory, although other mammalian signal sequences may be used to direct secretion of the protein, such as signal sequences from secreted polypeptides of the same or related species, as well as viral secretory leaders, for example, the herpes simplex glycoprotein D signal.

The DNA for such precursor region is preferably ligated in reading frame to DNA encoding Apo-2.

(ii) Origin of Replication Component

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2 $\mu$  plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used because it contains the early promoter).

Most expression vectors are "shuttle" vectors, i.e., they are capable of replication in at least one class of organisms but can be transfected into another organism for expression. For example, a vector is cloned in *E. coli* and then the same vector is transfected into yeast or mammalian cells for expression even though it is not capable of replicating independently of the host cell chromosome.

DNA may also be amplified by insertion into the host genome. This is readily accomplished using *Bacillus* species as hosts, for example, by including in the vector a DNA sequence that is complementary to a sequence found in *Bacillus* genomic DNA. Transfection of *Bacillus* with this vector results in homologous

recombination with the genome and insertion of Apo-2 DNA. However, the recovery of genomic DNA encoding Apo-2 is more complex than that of an exogenously replicated vector because restriction enzyme digestion is required to excise the Apo-2 DNA.

5 (iii) Selection Gene Component

Expression and cloning vectors typically contain a selection gene, also termed a selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not  
10 transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical  
15 nutrients not available from complex media, e.g., the gene encoding D-alanine racemase for *Bacilli*.

One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene produce a protein conferring  
20 drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin [Southern et al., J. Molec. Appl. Genet., 1:327 (1982)], mycophenolic acid (Mulligan et al., Science, 209:1422 (1980)) or hygromycin [Sugden et al., Mol. Cell. Biol., 5:410-413 (1985)]. The three examples given  
25 above employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug G418 or neomycin (geneticin), xgpt (mycophenolic acid), or hygromycin, respectively.

Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells  
30 competent to take up the Apo-2 nucleic acid, such as DHFR or thymidine kinase. The mammalian cell transformants are placed under selection pressure that only the transformants are uniquely adapted to survive by virtue of having taken up the marker. Selection pressure is imposed by culturing the transformants under  
35 conditions in which the concentration of selection agent in the medium is successively changed, thereby leading to amplification of both the selection gene and the DNA that encodes Apo-2. Amplification is the process by which genes in greater demand for the production of a protein critical for growth are reiterated in

tandem within the chromosomes of successive generations of recombinant cells. Increased quantities of Apo-2 are synthesized from the amplified DNA. Other examples of amplifiable genes include metallothionein-I and -II, adenosine deaminase, and  
5 ornithine decarboxylase.

Cells transformed with the DHFR selection gene may first be identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed  
10 is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub et al., Proc. Natl. Acad. Sci. USA, 77:4216 (1980). The transformed cells are then exposed to increased levels of methotrexate. This leads to the synthesis of multiple copies of the DHFR gene, and,  
15 concomitantly, multiple copies of other DNA comprising the expression vectors, such as the DNA encoding Apo-2. This amplification technique can be used with any otherwise suitable host, e.g., ATCC No. CCL61 CHO-K1, notwithstanding the presence of endogenous DHFR if, for example, a mutant DHFR gene that is highly  
20 resistant to Mtx is employed (EP 117,060).

Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding Apo-2, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3'-phosphotransferase  
25 (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, e.g., kanamycin, neomycin, or G418. See U.S. Patent No. 4,965,199.

A suitable selection gene for use in yeast is the *trp1* gene present in the yeast plasmid YRp7 [Stinchcomb et al., Nature, 282:39 (1979); Kingsman et al., Gene, 7:141 (1979); Tschemper et al., Gene, 10:157 (1980)]. The *trp1* gene provides a selection  
30 marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1 [Jones, Genetics, 85:12 (1977)]. The presence of the *trp1* lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, *Leu2*-deficient yeast strains (ATCC 20,622 or 38,626) are  
35 complemented by known plasmids bearing the *Leu2* gene.



In addition, vectors derived from the 1.6  $\mu$ m circular plasmid pKD1 can be used for transformation of *Kluyveromyces* yeasts [Bianchi et al., Curr. Genet., 12:185 (1987)]. More recently, an expression system for large-scale production of recombinant calf chymosin was reported for *K. lactis* [Van den Berg, Bio/Technology, 8:135 (1990)]. Stable multi-copy expression vectors for secretion of mature recombinant human serum albumin by industrial strains of *Kluyveromyces* have also been disclosed [Fleer et al., Bio/Technology, 9:968-975 (1991)].

10 (iv) Promoter Component

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the Apo-2 nucleic acid sequence. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of particular nucleic acid sequence, such as the Apo-2 nucleic acid sequence, to which they are operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, e.g., the presence or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These promoters are operably linked to Apo-2 encoding DNA by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector. Both the native Apo-2 promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of the Apo-2 DNA.

Promoters suitable for use with prokaryotic hosts include the  $\beta$ -lactamase and lactose promoter systems [Chang et al., Nature, 275:615 (1978); Goeddel et al., Nature, 281:544 (1979)], alkaline phosphatase, a tryptophan (trp) promoter system [Goeddel, Nucleic Acids Res., 8:4057 (1980); EP 36,776], and hybrid promoters such as the tac promoter [deBoer et al., Proc. Natl. Acad. Sci. USA, 80:21-25 (1983)]. However, other known bacterial promoters are suitable. Their nucleotide sequences have been published,

thereby enabling a skilled worker operably to ligate them to DNA encoding Apo-2 [Siebenlist et al., Cell, 20:269 (1980)] using linkers or adaptors to supply any required restriction sites. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding Apo-2.

Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CXCAAT region where X may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into eukaryotic expression vectors.

Examples of suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase [Hitzeman et al., J. Biol. Chem., 255:2073 (1980)] or other glycolytic enzymes [Hess et al., J. Adv. Enzyme Reg., 7:149 (1968); Holland, Biochemistry, 17:4900 (1978)], such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in EP 73,657. Yeast enhancers also are advantageously used with yeast promoters.

Apo-2 transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published 5 July 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40

(SV40), from heterologous mammalian promoters, e.g., the actin promoter or an immunoglobulin promoter, from heat-shock promoters, and from the promoter normally associated with the Apo-2 sequence, provided such promoters are compatible with the host cell systems.

5           The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication [Fiers et al., Nature, 273:113 (1978); Mulligan and Berg, Science, 209:1422-1427 (1980); Pavlakis et al., Proc. Natl. Acad. Sci. USA, 78:7398-7402 (1981)]. The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment [Greenaway et al., Gene, 18:355-360 (1982)]. A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. Patent No. 4,419,446. A  
10           modification of this system is described in U.S. Patent No. 4,601,978 [See also Gray et al., Nature, 295:503-508 (1982) on expressing cDNA encoding immune interferon in monkey cells; Reyes et al., Nature, 297:598-601 (1982) on expression of human  $\beta$ -interferon cDNA in mouse cells under the control of a thymidine  
20           kinase promoter from herpes simplex virus; Canaani and Berg, Proc. Natl. Acad. Sci. USA 79:5166-5170 (1982) on expression of the human interferon gene in cultured mouse and rabbit cells; and Gorman et al., Proc. Natl. Acad. Sci. USA, 79:6777-6781 (1982) on expression of bacterial CAT sequences in CV-1 monkey kidney cells, chicken  
25           embryo fibroblasts, Chinese hamster ovary cells, HeLa cells, and mouse NIH-3T3 cells using the Rous sarcoma virus long terminal repeat as a promoter].

(v) Enhancer Element Component

          Transcription of a DNA encoding the Apo-2 of this  
30           invention by higher eukaryotes may be increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp, that act on a promoter to increase its transcription. Enhancers are relatively orientation and position independent, having been found 5' [Laimins  
35           et al., Proc. Natl. Acad. Sci. USA, 78:993 (1981)] and 3' [Lusky et al., Mol. Cell Bio., 3:1108 (1983)] to the transcription unit, within an intron [Banerji et al., Cell, 33:729 (1983)], as well as within the coding sequence itself [Osborne et al., Mol. Cell Bio., 4:1293 (1984)]. Many enhancer sequences are now known from

mammalian genes (globin, elastase, albumin,  $\alpha$ -fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, Nature, 297:17-18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to the Apo-2 coding sequence, but is preferably located at a site 5' from the promoter.

(vi) Transcription Termination Component

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding Apo-2.

(vii) Construction and Analysis of Vectors

Construction of suitable vectors containing one or more of the above-listed components employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and re-ligated in the form desired to generate the plasmids required.

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures can be used to transform *E. coli* K12 strain 294 (ATCC 31,446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced by the method of Messing et al., Nucleic Acids Res., 9:309 (1981) or by the method of Maxim et al., Methods in Enzymology, 65:499 (1980).

(viii) Transient Expression Vectors

Expression vectors that provide for the transient expression in mammalian cells of DNA encoding Apo-2 may be employed. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host

cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector [Sambrook et al., supra]. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of such polypeptides for desired biological or physiological properties. Thus, transient expression systems are particularly useful in the invention for purposes of identifying Apo-2 variants.

(ix) Suitable Exemplary Vertebrate Cell Vectors

Other methods, vectors, and host cells suitable for adaptation to the synthesis of Apo-2 in recombinant vertebrate cell culture are described in Gething et al., Nature, 293:620-625 (1981); Mantei et al., Nature, 281:40-46 (1979); EP 117,060; and EP 117,058.

3. Selection and Transformation of Host Cells

Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include but are not limited to eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as *Escherichia*, e.g., *E. coli*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Proteus*, *Salmonella*, e.g., *Salmonella typhimurium*, *Serratia*, e.g., *Serratia marcescans*, and *Shigella*, as well as *Bacilli* such as *B. subtilis* and *B. licheniformis* (e.g., *B. licheniformis* 41P disclosed in DD 266,710 published 12 April 1989), *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*. Preferably, the host cell should secrete minimal amounts of proteolytic enzymes.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for Apo-2-encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein.

Suitable host cells for the expression of glycosylated Apo-2 are derived from multicellular organisms. Such host cells are capable of complex processing and glycosylation activities. In principle, any higher eukaryotic cell culture is workable, whether

from vertebrate or invertebrate culture. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified [See, e.g., Luckow et al., Bio/Technology, 6:47-55 (1988); Miller et al., in Genetic Engineering, Setlow et al., eds., Vol. 8 (Plenum Publishing, 1986), pp. 277-279; and Maeda et al., Nature, 315:592-594 (1985)]. A variety of viral strains for transfection are publicly available, e.g., the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV.

Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can be utilized as hosts. Typically, plant cells are transfected by incubation with certain strains of the bacterium *Agrobacterium tumefaciens*. During incubation of the plant cell culture with *A. tumefaciens*, the DNA encoding the Apo-2 can be transferred to the plant cell host such that it is transfected, and will, under appropriate conditions, express the Apo-2-encoding DNA. In addition, regulatory and signal sequences compatible with plant cells are available, such as the nopaline synthase promoter and polyadenylation signal sequences [Depicker et al., J. Mol. Appl. Gen., 1:561 (1982)]. In addition, DNA segments isolated from the upstream region of the T-DNA 780 gene are capable of activating or increasing transcription levels of plant-expressible genes in recombinant DNA-containing plant tissue [EP 321,196 published 21 June 1989].

Propagation of vertebrate cells in culture (tissue culture) is also well known in the art [See, e.g., Tissue Culture, Academic Press, Kruse and Patterson, editors (1973)]. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., J. Gen. Virol., 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, Proc. Natl. Acad. Sci. USA, 77:4216 (1980)); mouse sertoli cells (TM4, Mather, Biol. Reprod., 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical

carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells  
5 (Mather et al., Annals N.Y. Acad. Sci., 383:44-68 (1982)); MRC 5 cells; and FS4 Cells.

Host cells are transfected and preferably transformed with the above-described expression or cloning vectors for Apo-2 production and cultured in conventional nutrient media modified as  
10 appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the  
15 ordinarily skilled artisan, for example,  $\text{CaPO}_4$  and electroporation. Successful transfection is generally recognized when any indication of the operation of this vector occurs within the host cell.

Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element  
20 or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in Sambrook et al., supra, or electroporation is generally used for prokaryotes or other cells that contain  
25 substantial cell-wall barriers. Infection with *Agrobacterium tumefaciens* is used for transformation of certain plant cells, as described by Shaw et al., Gene, 23:315 (1983) and WO 89/05859 published 29 June 1989. In addition, plants may be transfected using ultrasound treatment as described in WO 91/00358 published 10  
30 January 1991.

For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb, Virology, 52:456-457 (1978) is preferred. General aspects of mammalian cell host system transformations have been described in U.S. Pat. No.  
35 4,399,216. Transformations into yeast are typically carried out according to the method of Van Solingen et al., J. Bact., 130:946 (1977) and Hsiao et al., Proc. Natl. Acad. Sci. (USA), 76:3829 (1979). However, other methods for introducing DNA into cells, such as by nuclear microinjection, electroporation, bacterial

protoplast fusion with intact cells, or polycations, e.g., polybrene, polyornithine, may also be used. For various techniques for transforming mammalian cells, see Keown et al., Methods in Enzymology, 185:527-537 (1990) and Mansour et al., Nature, 336:348-352 (1988).

#### 4. Culturing the Host Cells

Prokaryotic cells used to produce Apo-2 may be cultured in suitable media as described generally in Sambrook et al., supra.

The mammalian host cells used to produce Apo-2 may be cultured in a variety of media. Examples of commercially available media include Ham's F10 (Sigma), Minimal Essential Medium ("MEM", Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ("DMEM", Sigma). Any such media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin<sup>TM</sup> drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

In general, principles, protocols, and practical techniques for maximizing the productivity of mammalian cell cultures can be found in Mammalian Cell Biotechnology: a Practical Approach, M. Butler, ed. (IRL Press, 1991).

The host cells referred to in this disclosure encompass cells in culture as well as cells that are within a host animal.

#### 5. Detecting Gene Amplification/Expression

Gene amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA [Thomas, Proc. Natl. Acad. Sci. USA, 77:5201-5205 (1980)], dot blotting (DNA analysis), or *in situ* hybridization, using an appropriately labeled probe, based on the sequences provided herein. Various labels may be employed, most commonly radioisotopes, and particularly <sup>32</sup>P.



However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionucleotides, fluorescers or enzymes. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of cells or tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. With immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and fixation, followed by reaction with labeled antibodies specific for the gene product coupled, where the labels are usually visually detectable, such as enzymatic labels, fluorescent labels, or luminescent labels.

Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared in any mammal. Conveniently, the antibodies may be prepared against a native sequence Apo-2 polypeptide or against a synthetic peptide based on the DNA sequences provided herein or against exogenous sequence fused to Apo-2 DNA and encoding a specific antibody epitope.

#### 6. Purification of Apo-2 Polypeptide

Forms of Apo-2 may be recovered from culture medium or from host cell lysates. If the Apo-2 is membrane-bound, it can be released from the membrane using a suitable detergent solution (e.g. Triton-X 100) or its extracellular domain may be released by enzymatic cleavage.

When Apo-2 is produced in a recombinant cell other than one of human origin, the Apo-2 is free of proteins or polypeptides of human origin. However, it may be desired to purify Apo-2 from recombinant cell proteins or polypeptides to obtain preparations that are substantially homogeneous as to Apo-2. As a first step, the culture medium or lysate may be centrifuged to remove

particulate cell debris. Apo-2 thereafter is purified from contaminant soluble proteins and polypeptides, with the following procedures being exemplary of suitable purification procedures: by fractionation on an ion-exchange column; ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; and protein A Sepharose columns to remove contaminants such as IgG.

Apo-2 variants in which residues have been deleted, inserted, or substituted can be recovered in the same fashion as native sequence Apo-2, taking account of changes in properties occasioned by the variation. For example, preparation of an Apo-2 fusion with another protein or polypeptide, e.g., a bacterial or viral antigen, immunoglobulin sequence, or receptor sequence, may facilitate purification; an immunoaffinity column containing antibody to the sequence can be used to adsorb the fusion polypeptide. Other types of affinity matrices also can be used.

A protease inhibitor such as phenyl methyl sulfonyl fluoride (PMSF) also may be useful to inhibit proteolytic degradation during purification, and antibiotics may be included to prevent the growth of adventitious contaminants. One skilled in the art will appreciate that purification methods suitable for native sequence Apo-2 may require modification to account for changes in the character of Apo-2 or its variants upon expression in recombinant cell culture.

#### 7. Covalent Modifications of Apo-2 Polypeptides

Covalent modifications of Apo-2 are included within the scope of this invention. One type of covalent modification of the Apo-2 is introduced into the molecule by reacting targeted amino acid residues of the Apo-2 with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues of the Apo-2.

Derivatization with bifunctional agents is useful for crosslinking Apo-2 to a water-insoluble support matrix or surface for use in the method for purifying anti-Apo-2 antibodies, and vice-versa. Derivatization with one or more bifunctional agents will also be useful for crosslinking Apo-2 molecules to generate Apo-2 dimers. Such dimers may increase binding avidity and extend

half-life of the molecule in vivo. Commonly used crosslinking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including  
5 disuccinimidyl esters such as 3,3'-dithiobis(succinimidyl-propionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)-dithio]propioimide yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light.  
10 Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Patent Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

15 Other modifications include deamidation of glutamyl and asparagyl residues to the corresponding glutamyl and aspartyl residues, respectively, hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the  $\alpha$ -amino groups of lysine, arginine, and  
20 histidine side chains [T.E. Creighton, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)], acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group. The modified forms of the residues fall within the scope of the present invention.

25 Another type of covalent modification of the Apo-2 polypeptide included within the scope of this invention comprises altering the native glycosylation pattern of the polypeptide. "Altering the native glycosylation pattern" is intended for purposes herein to mean deleting one or more carbohydrate moieties  
30 found in native sequence Apo-2, and/or adding one or more glycosylation sites that are not present in the native sequence Apo-2.

Glycosylation of polypeptides is typically either N-linked or O-linked. N-linked refers to the attachment of the  
35 carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either

of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-aceylgalactosamine, galactose, or xylose to a hydroxylamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Addition of glycosylation sites to the Apo-2 polypeptide may be accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the native sequence Apo-2 (for O-linked glycosylation sites). The Apo-2 amino acid sequence may optionally be altered through changes at the DNA level, particularly by mutating the DNA encoding the Apo-2 polypeptide at preselected bases such that codons are generated that will translate into the desired amino acids. The DNA mutation(s) may be made using methods described above and in U.S. Pat. No. 5,364,934, supra.

Another means of increasing the number of carbohydrate moieties on the Apo-2 polypeptide is by chemical or enzymatic coupling of glycosides to the polypeptide. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. These methods are described in WO 87/05330 published 11 September 1987, and in Aplin and Wriston, CRC Crit. Rev. Biochem., pp. 259-306 (1981).

Removal of carbohydrate moieties present on the Apo-2 polypeptide may be accomplished chemically or enzymatically or by mutational substitution of codons encoding for amino acid residues that serve as targets for glycosylation. For instance, chemical deglycosylation by exposing the polypeptide to the compound trifluoromethanesulfonic acid, or an equivalent compound can result in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the polypeptide intact. Chemical deglycosylation is described by Hakimuddin, et al., Arch. Biochem. Biophys., 259:52 (1987) and by Edge et al., Anal. Biochem., 118:131 (1981). Enzymatic cleavage of

carbohydrate moieties on polypeptides can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura et al., Meth. Enzymol., 138:350 (1987).

5 Glycosylation at potential glycosylation sites may be prevented by the use of the compound tunicamycin as described by Duksin et al., J. Biol. Chem., 257:3105 (1982). Tunicamycin blocks the formation of protein-N-glycoside linkages.

Another type of covalent modification of Apo-2 comprises linking the Apo-2 polypeptide to one of a variety of  
10 nonproteinaceous polymers, e.g., polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

#### 8. Apo-2 Chimeras

15 The present invention also provides chimeric molecules comprising Apo-2 fused to another, heterologous polypeptide or amino acid sequence.

In one embodiment, the chimeric molecule comprises a fusion of the Apo-2 with a tag polypeptide which provides an  
20 epitope to which an anti-tag antibody can selectively bind. The epitope tag is generally placed at the amino- or carboxyl- terminus of the Apo-2. The presence of such epitope-tagged forms of the Apo-2 can be detected using an antibody against the tag polypeptide. Also, provision of the epitope tag enables the Apo-2  
25 to be readily purified by affinity purification using an anti-tag antibody or another type of affinity matrix that binds to the epitope tag.

Various tag polypeptides and their respective antibodies are well known in the art. Examples include the flu HA tag  
30 polypeptide and its antibody 12CA5 [Field et al., Mol. Cell. Biol., 8:2159-2165 (1988)]; the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto [Evan et al., Molecular and Cellular Biology, 5:3610-3616 (1985)]; and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody [Paborsky et al., Protein Engineering, 3(6):547-553 (1990)]. Other tag polypeptides include  
35 the Flag-peptide [Hopp et al., BioTechnology, 6:1204-1210 (1988)]; the KT3 epitope peptide [Martin et al., Science, 255:192-194 (1992)]; an  $\alpha$ -tubulin epitope peptide [Skinner et al., J. Biol. Chem., 266:15163-15166 (1991)]; and the T7 gene 10 protein peptide

tag [Lutz-Freyermuth et al., Proc. Natl. Acad. Sci. USA, 87:6393-6397 (1990)]. Once the tag polypeptide has been selected, an antibody thereto can be generated using the techniques disclosed herein.

5           Generally, epitope-tagged Apo-2 may be constructed and produced according to the methods described above. Epitope-tagged Apo-2 is also described in the Examples below. Apo-2-tag polypeptide fusions are preferably constructed by fusing the cDNA sequence encoding the Apo-2 portion in-frame to the tag polypeptide  
10 DNA sequence and expressing the resultant DNA fusion construct in appropriate host cells. Ordinarily, when preparing the Apo-2-tag polypeptide chimeras of the present invention, nucleic acid encoding the Apo-2 will be fused at its 3' end to nucleic acid encoding the N-terminus of the tag polypeptide, however 5' fusions  
15 are also possible. For example, a polyhistidine sequence of about 5 to about 10 histidine residues may be fused at the N- terminus or the C- terminus and used as a purification handle in affinity chromatography.

          Epitope-tagged Apo-2 can be purified by affinity  
20 chromatography using the anti-tag antibody. The matrix to which the affinity antibody is attached may include, for instance, agarose, controlled pore glass or poly(styrenedivinyl)benzene. The epitope-tagged Apo-2 can then be eluted from the affinity column using techniques known in the art.

25           In another embodiment, the chimeric molecule comprises an Apo-2 polypeptide fused to an immunoglobulin sequence. The chimeric molecule may also comprise a particular domain sequence of Apo-2, such as an extracellular domain sequence of Apo-2 fused to an immunoglobulin sequence. This includes chimeras in monomeric,  
30 homo- or heteromultimeric, and particularly homo- or heterodimeric, or -tetrameric forms; optionally, the chimeras may be in dimeric forms or homodimeric heavy chain forms. Generally, these assembled immunoglobulins will have known unit structures as represented by the following diagrams.

X or A

\\_\_\_\_\_C<sub>H</sub> or C<sub>L</sub>

5

X or A

\\_\_\_\_\_Y\_\_\_\_\_C<sub>H</sub> or C<sub>L</sub>

10

A

A \\_\_\_\_\_C<sub>L</sub>\\_\_\_\_\_C<sub>H</sub>

15

A

V<sub>H</sub> \\_\_\_\_\_C<sub>L</sub>\\_\_\_\_\_C<sub>H</sub>

20

V<sub>L</sub>A \\_\_\_\_\_C<sub>L</sub>\\_\_\_\_\_C<sub>H</sub>

25

X

A \\_\_\_\_\_C<sub>L</sub>\\_\_\_\_\_C<sub>H</sub>

30

A

X \\_\_\_\_\_C<sub>L</sub>\\_\_\_\_\_C<sub>H</sub>

A basic four chain structural unit is the form in which  
 35 IgG, IgD, and IgE exist. A four chain unit is repeated in the  
 higher molecular weight immunoglobulins; IgM generally exists as a  
 pentamer of basic four-chain units held together by disulfide  
 bonds. IgA globulin, and occasionally IgG globulin, may also exist

in a multimeric form in serum. In the case of multimers, each four chain unit may be the same or different.

The following diagrams depict some exemplary monomer, homo- and heterodimer and homo- and heteromultimer structures.

5 These diagrams are merely illustrative, and the chains of the multimers are believed to be disulfide bonded in the same fashion as native immunoglobulins.



monomer: A \_\_\_\_\_ C<sub>L</sub> or C<sub>H</sub>

5 homodimer: A  
 \ \_\_\_\_\_ C<sub>L</sub> or C<sub>H</sub>  
 \_\_\_\_\_ C<sub>L</sub> or C<sub>H</sub>  
 /  
 A

10

heterodimer: A  
 \ \_\_\_\_\_ C<sub>L</sub> or C<sub>H</sub>  
 \_\_\_\_\_ C<sub>L</sub> or C<sub>H</sub>  
 /  
 X

15

homotetramer: A  
 A \ \_\_\_\_\_ C<sub>L</sub>  
 \ \_\_\_\_\_ C<sub>L</sub> or C<sub>H</sub>  
 \_\_\_\_\_ C<sub>L</sub> or C<sub>H</sub>  
 / \_\_\_\_\_ C<sub>L</sub>  
 A /  
 A

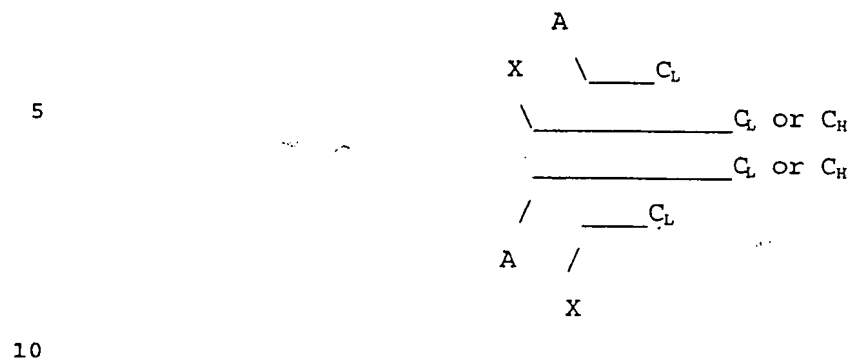
20

25

heterotetramer: A  
 A \ \_\_\_\_\_ C<sub>L</sub>  
 \ \_\_\_\_\_ C<sub>L</sub> or C<sub>H</sub>  
 \_\_\_\_\_ C<sub>L</sub> or C<sub>H</sub>  
 / \_\_\_\_\_ C<sub>L</sub>  
 X /  
 X

30

and



In the foregoing diagrams, "A" means an Apo-2 sequence or an Apo-2 sequence fused to a heterologous sequence; X is an additional agent, which may be the same as A or different, a portion of an immunoglobulin superfamily member such as a variable region or a variable region-like domain, including a native or chimeric immunoglobulin variable region, a toxin such as pseudomonas exotoxin or ricin, or a sequence functionally binding to another protein, such as other cytokines (i.e., IL-1, interferon- $\gamma$ ) or cell surface molecules (i.e., NGFR, CD40, OX40, Fas antigen, T2 proteins of Shope and myxoma poxviruses), or a polypeptide therapeutic agent not otherwise normally associated with a constant domain; Y is a linker or another receptor sequence; and  $V_L$ ,  $V_H$ ,  $C_L$  and  $C_H$  represent light or heavy chain variable or constant domains of an immunoglobulin. Structures comprising at least one CRD of an Apo-2 sequence as "A" and another cell-surface protein having a repetitive pattern of CRDs (such as TNFR) as "X" are specifically included.

It will be understood that the above diagrams are merely exemplary of the possible structures of the chimeras of the present invention, and do not encompass all possibilities. For example, there might desirably be several different "A"s, "X"s, or "Y"s in any of these constructs. Also, the heavy or light chain constant domains may be originated from the same or different immunoglobulins. All possible permutations of the illustrated and similar structures are all within the scope of the invention herein.

In general, the chimeric molecules can be constructed in a fashion similar to chimeric antibodies in which a variable domain

from an antibody of one species is substituted for the variable domain of another species. See, for example, EP 0 125 023; EP 173,494; Munro, Nature, 312:597 (13 December 1984); Neuberger et al., Nature, 312:604-608 (13 December 1984); Sharon et al., Nature, 309:364-367 (24 May 1984); Morrison et al., Proc. Nat'l. Acad. Sci. USA, 81:6851-6855 (1984); Morrison et al., Science, 229:1202-1207 (1985); Boulianne et al., Nature, 312:643-646 (13 December 1984); Capon et al., Nature, 337:525-531 (1989); Traunecker et al., Nature, 339:68-70 (1989).

Alternatively, the chimeric molecules may be constructed as follows. The DNA including a region encoding the desired sequence, such as an Apo-2 and/or TNFR sequence, is cleaved by a restriction enzyme at or proximal to the 3' end of the DNA encoding the immunoglobulin-like domain(s) and at a point at or near the DNA encoding the N-terminal end of the Apo-2 or TNFR polypeptide (where use of a different leader is contemplated) or at or proximal to the N-terminal coding region for TNFR (where the native signal is employed). This DNA fragment then is readily inserted proximal to DNA encoding an immunoglobulin light or heavy chain constant region and, if necessary, the resulting construct tailored by deletional mutagenesis. Preferably, the Ig is a human immunoglobulin when the chimeric molecule is intended for *in vivo* therapy for humans. DNA encoding immunoglobulin light or heavy chain constant regions is known or readily available from cDNA libraries or is synthesized. See for example, Adams et al., Biochemistry, 19:2711-2719 (1980); Gough et al., Biochemistry, 19:2702-2710 (1980); Dolby et al., Proc. Natl. Acad. Sci. USA, 77:6027-6031 (1980); Rice et al., Proc. Natl. Acad. Sci., 79:7862-7865 (1982); Falkner et al., Nature, 298:286-288 (1982); and Morrison et al., Ann. Rev. Immunol., 2:239-256 (1984).

Further details of how to prepare such fusions are found in publications concerning the preparation of immunoadhesins. Immunoadhesins in general, and CD4-Ig fusion molecules specifically are disclosed in WO 89/02922, published 6 April 1989. Molecules comprising the extracellular portion of CD4, the receptor for human immunodeficiency virus (HIV), linked to IgG heavy chain constant region are known in the art and have been found to have a markedly longer half-life and lower clearance than the soluble extracellular portion of CD4 [Capon et al., *supra*; Byrn et al., Nature, 344:667

(1990)]. The construction of specific chimeric TNFR-IgG molecules is also described in Ashkenazi et al. Proc. Natl. Acad. Sci., 88:10535-10539 (1991); Lesslauer et al. [J. Cell. Biochem. Supplement 15F, 1991, p. 115 (P 432)]; and Peppel and Beutler, J. Cell. Biochem. Supplement 15F, 1991, p. 118 (P 439)].

B. Therapeutic and Non-therapeutic Uses for Apo-2

Apo-2, as disclosed in the present specification, can be employed therapeutically to induce apoptosis in mammalian cells. This therapy can be accomplished for instance, using *in vivo* or *ex vivo* gene therapy techniques and includes the use of the death domain sequences disclosed herein. The Apo-2 chimeric molecules (including the chimeric molecules containing an extracellular domain sequence of Apo-2) comprising immunoglobulin sequences can also be employed therapeutically to inhibit apoptosis or NF-KB induction by Apo-2L or by another ligand that Apo-2 binds to.

The Apo-2 of the invention also has utility in non-therapeutic applications. Nucleic acid sequences encoding the Apo-2 may be used as a diagnostic for tissue-specific typing. For example, procedures like *in situ* hybridization, Northern and Southern blotting, and PCR analysis may be used to determine whether DNA and/or RNA encoding Apo-2 is present in the cell type(s) being evaluated. Apo-2 nucleic acid will also be useful for the preparation of Apo-2 by the recombinant techniques described herein.

The isolated Apo-2 may be used in quantitative diagnostic assays as a control against which samples containing unknown quantities of Apo-2 may be prepared. Apo-2 preparations are also useful in generating antibodies, as standards in assays for Apo-2 (e.g., by labeling Apo-2 for use as a standard in a radioimmunoassay, radioreceptor assay, or enzyme-linked immunoassay), in affinity purification techniques, and in competitive-type receptor binding assays when labeled with, for instance, radioiodine, enzymes, or fluorophores.

Modified forms of the Apo-2, such as the Apo-2-IgG chimeric molecules (immunoadhesins) described above, can be used as immunogens in producing anti-Apo-2 antibodies.

Nucleic acids which encode Apo-2 or its modified forms can also be used to generate either transgenic animals or "knock

out" animals which, in turn, are useful in the development and screening of therapeutically useful reagents. A transgenic animal (e.g., a mouse or rat) is an animal having cells that contain a transgene, which transgene was introduced into the animal or an ancestor of the animal at a prenatal, e.g., an embryonic stage. A transgene is a DNA which is integrated into the genome of a cell from which a transgenic animal develops. In one embodiment, cDNA encoding Apo-2 or an appropriate sequence thereof (such as Apo-2-IgG) can be used to clone genomic DNA encoding Apo-2 in accordance with established techniques and the genomic sequences used to generate transgenic animals that contain cells which express DNA encoding Apo-2. Methods for generating transgenic animals, particularly animals such as mice or rats, have become conventional in the art and are described, for example, in U.S. Patent Nos. 4,736,866 and 4,870,009. Typically, particular cells would be targeted for Apo-2 transgene incorporation with tissue-specific enhancers. Transgenic animals that include a copy of a transgene encoding Apo-2 introduced into the germ line of the animal at an embryonic stage can be used to examine the effect of increased expression of DNA encoding Apo-2. Such animals can be used as tester animals for reagents thought to confer protection from, for example, pathological conditions associated with excessive apoptosis. In accordance with this facet of the invention, an animal is treated with the reagent and a reduced incidence of the pathological condition, compared to untreated animals bearing the transgene, would indicate a potential therapeutic intervention for the pathological condition. In another embodiment, transgenic animals that carry a soluble form of Apo-2 such as an Apo-2 ECD or an immunoglobulin chimera of such form could be constructed to test the effect of chronic neutralization of Apo-2L, a ligand of Apo-2.

Alternatively, non-human homologues of Apo-2 can be used to construct an Apo-2 "knock out" animal which has a defective or altered gene encoding Apo-2 as a result of homologous recombination between the endogenous gene encoding Apo-2 and altered genomic DNA encoding Apo-2 introduced into an embryonic cell of the animal. For example, cDNA encoding Apo-2 can be used to clone genomic DNA encoding Apo-2 in accordance with established techniques. A portion of the genomic DNA encoding Apo-2 can be deleted or replaced with another gene, such as a gene encoding a selectable

marker which can be used to monitor integration. Typically, several kilobases of unaltered flanking DNA (both at the 5' and 3' ends) are included in the vector [see e.g., Thomas and Capecchi, Cell, 51:503 (1987) for a description of homologous recombination vectors]. The vector is introduced into an embryonic stem cell line (e.g., by electroporation) and cells in which the introduced DNA has homologously recombined with the endogenous DNA are selected [see e.g., Li et al., Cell, 69:915 (1992)]. The selected cells are then injected into a blastocyst of an animal (e.g., a mouse or rat) to form aggregation chimeras [see e.g., Bradley, in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E. J. Robertson, ed. (IRL, Oxford, 1987), pp. 113-152]. A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term to create a "knock out" animal. Progeny harboring the homologously recombined DNA in their germ cells can be identified by standard techniques and used to breed animals in which all cells of the animal contain the homologously recombined DNA. Knockout animals can be characterized for instance, for their ability to defend against certain pathological conditions and for their development of pathological conditions due to absence of the Apo-2 polypeptide, including for example, development of tumors.

### C. Anti-Apo-2 Antibody Preparation

The present invention further provides anti-Apo-2 antibodies. Antibodies against Apo-2 may be prepared as follows. Exemplary antibodies include polyclonal, monoclonal, humanized, bispecific, and heteroconjugate antibodies.

#### 1. Polyclonal Antibodies

The Apo-2 antibodies may comprise polyclonal antibodies. Methods of preparing polyclonal antibodies are known to the skilled artisan. Polyclonal antibodies can be raised in a mammal, for example, by one or more injections of an immunizing agent and, if desired, an adjuvant. Typically, the immunizing agent and/or adjuvant will be injected in the mammal by multiple subcutaneous or intraperitoneal injections. The immunizing agent may include the Apo-2 polypeptide or a fusion protein thereof. An example of a suitable immunizing agent is an Apo-2-IgG fusion protein, such as an Apo-2 ECD-IgG fusion protein. Cells expressing Apo-2 at their

surface may also be employed. It may be useful to conjugate the immunizing agent to a protein known to be immunogenic in the mammal being immunized. Examples of such immunogenic proteins which may be employed include but are not limited to keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, and soybean trypsin inhibitor. An aggregating agent such as alum may also be employed to enhance the mammal's immune response. Examples of adjuvants which may be employed include Freund's complete adjuvant and MPL-TDM adjuvant (monophosphoryl Lipid A, synthetic trehalose dicorynomycolate). The immunization protocol may be selected by one skilled in the art without undue experimentation. The mammal can then be bled, and the serum assayed for antibody titer. If desired, the mammal can be boosted until the antibody titer increases or plateaus.

## 2. Monoclonal Antibodies

The Apo-2 antibodies may, alternatively, be monoclonal antibodies. Monoclonal antibodies may be prepared using hybridoma methods, such as those described by Kohler and Milstein, supra. In a hybridoma method, a mouse, hamster, or other appropriate host animal, is typically immunized (such as described above) with an immunizing agent to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the immunizing agent. Alternatively, the lymphocytes may be immunized *in vitro*.

The immunizing agent will typically include the Apo-2 polypeptide or a fusion protein thereof. An example of a suitable immunizing agent is an Apo-2-IgG fusion protein or chimeric molecule. A specific example of an Apo-2 ECD-IgG immunogen is described in Example 9 below. Cells expressing Apo-2 at their surface may also be employed. Generally, either peripheral blood lymphocytes ("PBLs") are used if cells of human origin are desired, or spleen cells or lymph node cells are used if non-human mammalian sources are desired. The lymphocytes are then fused with an immortalized cell line using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell [Goding, Monoclonal Antibodies: Principles and Practice, Academic Press, (1986) pp. 59-103]. Immortalized cell lines are usually transformed mammalian cells, particularly myeloma cells of rodent, bovine and human origin. Usually, rat or mouse myeloma cell lines are employed.

The hybridoma cells may be cultured in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, immortalized cells. For example, if the parental transformed cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine ("HAT medium"), which substances prevent the growth of HGPRT-deficient cells.

Preferred immortalized cell lines are those that fuse efficiently, support stable high level expression of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. More preferred immortalized cell lines are murine myeloma lines, which can be obtained, for instance, from the Salk Institute Cell Distribution Center, San Diego, California and the American Type Culture Collection, Manassas, Virginia. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies [Kozbor, J. Immunol., 133:3001 (1984); Brodeur et al., Monoclonal Antibody Production Techniques and Applications, Marcel Dekker, Inc., New York, (1987) pp. 51-63].

The culture medium in which the hybridoma cells are cultured can then be assayed for the presence of monoclonal antibodies directed against Apo-2. Preferably, the binding specificity of monoclonal antibodies produced by the hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA). Such techniques and assays are known in the art. The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson and Pollard, Anal. Biochem., 107:220 (1980).

After the desired hybridoma cells are identified, the clones may be subcloned by limiting dilution procedures and grown by standard methods [Goding, supra]. Suitable culture media for this purpose include, for example, Dulbecco's Modified Eagle's Medium and RPMI-1640 medium. Alternatively, the hybridoma cells may be grown *in vivo* as ascites in a mammal.

The monoclonal antibodies secreted by the subclones may be isolated or purified from the culture medium or ascites fluid by conventional immunoglobulin purification procedures such as, for



example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

The monoclonal antibodies may also be made by recombinant DNA methods, such as those described in U.S. Patent No. 4,816,567. DNA encoding the monoclonal antibodies of the invention can be readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells of the invention serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. The DNA also may be modified, for example, by substituting the coding sequence for human heavy and light chain constant domains in place of the homologous murine sequences [U.S. Patent No. 4,816,567; Morrison et al., *supra*] or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. Such a non-immunoglobulin polypeptide can be substituted for the constant domains of an antibody of the invention, or can be substituted for the variable domains of one antigen-combining site of an antibody of the invention to create a chimeric bivalent antibody.

As described in the Examples below, anti-Apo-2 monoclonal antibodies have been prepared. One of these antibodies, 3F11.39.7, has been deposited with ATCC and has been assigned deposit accession no. HB-12456. In one embodiment, the monoclonal antibodies of the invention will have the same biological characteristics as the monoclonal antibodies secreted by the hybridoma cell line(s) deposited under Accession No. HB-12456. The term "biological characteristics" is used to refer to the *in vitro* and/or *in vivo* activities or properties of the monoclonal antibody, such as the ability to specifically bind to Apo-2 or to substantially block, induce or enhance Apo-2 activation. As disclosed in the present specification, the 3F11.39.7 monoclonal antibody (HB-12456) is characterized as having agonistic activity for inducing apoptosis, binding to the Apo-2 receptor, having blocking activity as described in the Examples below, and having

some cross-reactivity to DR4 but not to DcR1 or DcR2. Optionally, the monoclonal antibody will bind to the same epitope as the 3F11.39.7 antibody disclosed herein. This can be determined by conducting various assays, such as described herein and in the Examples. For instance, to determine whether a monoclonal antibody has the same specificity as the 3F11.39.7 antibody specifically disclosed, one can compare activity in Apo-2 blocking and apoptosis induction assays, such as those described in the Examples below.

The antibodies of the invention may also comprise monovalent antibodies. Methods for preparing monovalent antibodies are well known in the art. For example, one method involves recombinant expression of immunoglobulin light chain and modified heavy chain. The heavy chain is truncated generally at any point in the Fc region so as to prevent heavy chain crosslinking. Alternatively, the relevant cysteine residues are substituted with another amino acid residue or are deleted so as to prevent crosslinking.

In vitro methods are also suitable for preparing monovalent antibodies. Digestion of antibodies to produce fragments thereof, particularly, Fab fragments, can be accomplished using routine techniques known in the art. For instance, digestion can be performed using papain. Examples of papain digestion are described in WO 94/29348 published 12/22/94 and U.S. Patent No. 4,342,566. Papain digestion of antibodies typically produces two identical antigen binding fragments, called Fab fragments, each with a single antigen binding site, and a residual Fc fragment. Pepsin treatment yields an  $F(ab')_2$  fragment that has two antigen combining sites and is still capable of cross-linking antigen.

The Fab fragments produced in the antibody digestion also contain the constant domains of the light chain and the first constant domain ( $CH_1$ ) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain  $CH_1$  domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group.  $F(ab')_2$  antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

### 3. Humanized Antibodies

The Apo-2 antibodies of the invention may further comprise humanized antibodies or human antibodies. Humanized forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab')<sub>2</sub>, or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin [Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature, 332:323-329 (1988); and Presta, Curr. Op. Struct. Biol., 2:593-596 (1992)].

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers [Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature, 332:323-327 (1988); Verhoeyen et al., Science, 239:1534-1536 (1988)], by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Patent No. 4,816,567),

wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important in order to reduce antigenicity. According to the "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody [Sims et al., J. Immunol., 151:2296 (1993); Chothia and Lesk, J. Mol. Biol., 196:901 (1987)]. Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies [Carter et al., Proc. Natl. Acad. Sci. USA, 89:4285 (1992); Presta et al., J. Immunol., 151:2623 (1993)].

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three dimensional models of the parental and humanized sequences. Three dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the consensus and import sequence so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in

influencing antigen binding [see, WO 94/04679 published 3 March 1994].

Transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production can be employed. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge [see, e.g., Jakobovits et al., Proc. Natl. Acad. Sci. USA, 90:2551-255 (1993); Jakobovits et al., Nature, 362:255-258 (1993); Bruggemann et al., Year in Immuno., 7:33 (1993)].

Human antibodies can also be produced in phage display libraries [Hoogenboom and Winter, J. Mol. Biol., 227:381 (1992); Marks et al., J. Mol. Biol., 222:581 (1991)]. The techniques of Cole et al. and Boerner et al. are also available for the preparation of human monoclonal antibodies (Cole et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985) and Boerner et al., J. Immunol., 147(1):86-95 (1991)]. Suitable methods for preparing phage libraries have been reviewed and are described in Winter et al., Annu. Rev. Immunol., 12:433-55 (1994); Soderlind et al., Immunological Reviews, 130:109-123 (1992); Hoogenboom, Tibtech February 1997, Vol. 15; Neri et al., Cell Biophysics, 27:47-61 (1995). Libraries of single chain antibodies may also be prepared by the methods described in WO 92/01047, WO 92/20791, WO 93/06213, WO 93/11236, WO 93/19172, WO 95/01438 and WO 95/15388. Antibody libraries are also commercially available, for example, from Cambridge Antibody Technologies (C.A.T.), Cambridge, UK. Binding selection against an antigen, in this case Apo-2, can be carried out as described in greater detail in the Examples below.

As described in the Examples below, anti-Apo-2 single-chain Fv (scFv) antibodies have been identified using a phage display library. Three of these antibodies, referred to herein as 16E2, 24C4 and 20E6, have been sequenced and characterized. The respective DNA and amino acid sequences and complementarity determining regions of these antibodies are shown in Figures 15A-15C and 16. In one embodiment of the invention, scFv Apo-2 antibodies will have the same biological characteristics as the 16E2, 24C4 or 20E6 antibodies identified herein. The term

"biological characteristics" is used to refer to the *in vitro* and/or *in vivo* activities or properties of the scFv antibody, such as the ability to specifically bind to Apo-2 or to substantially induce or enhance Apo-2 activation. As disclosed in the present specification, the 16E2, 24C4 and 20E6 antibodies are characterized as binding to Apo-2, having agonistic activity for inducing apoptosis, and having no cross-reactivity to DR4 or several of the other known molecules recognized by the Apo-2 ligand. Optionally, the scFv Apo-2 antibody will bind to the same epitope or epitopes recognized by the 16E2, 24C4 or 20E6 antibodies disclosed herein. This can be determined by conducting various assays, such as described herein and in the Examples. For instance, to determine whether a scFv antibody has the same specificity as the 16E2, 24C4 or 20E6 antibodies specifically disclosed, one can compare activity in apoptosis induction assays, such as those described in the Examples below.

Optionally the scFv antibodies to Apo-2 may include antibodies which contain a VH and VL chain that include one or more complementarity determining region (CDR) amino acid sequences identified in Figure 16 for the 16E2, 20E6, or 24C4 antibodies.

#### 4. Bispecific Antibodies

Bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens. In the present case, one of the binding specificities is for the Apo-2, the other one is for any other antigen, and preferably for a cell-surface protein or receptor or receptor subunit.

Methods for making bispecific antibodies are known in the art. Traditionally, the recombinant production of bispecific antibodies is based on the co-expression of two immunoglobulin heavy-chain/light-chain pairs, where the two heavy chains have different specificities [Milstein and Cuello, Nature, 305:537-539 (1983)]. Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of ten different antibody molecules, of which only one has the correct bispecific structure. The purification of the correct molecule is usually accomplished by affinity chromatography steps.

Similar procedures are disclosed in WO 93/08829, published 13 May 1993, and in Traunecker et al., EMBO J., 10:3655-3659 (1991).

According to a different and more preferred approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy-chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light-chain binding present in at least one of the fusions. DNAs encoding the immunoglobulin heavy-chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance. In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy-chain/light-chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690 published 3 March 1994. For further details of generating bispecific antibodies see, for example, Suresh et al., Methods in Enzymology, 121:210 (1986).

#### 5. Heteroconjugate Antibodies

Heteroconjugate antibodies are also within the scope of the present invention. Heteroconjugate antibodies are composed of two covalently joined antibodies. Such antibodies have, for example, been proposed to target immune system cells to unwanted

cells [US Patent No. 4,676,980], and for treatment of HIV infection [WO 91/00360; WO 92/200373; EP 03089]. It is contemplated that the antibodies may be prepared *in vitro* using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins may be constructed using a disulfide exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptobutyrimidate and those disclosed, for example, in U.S. Pat. No. 4,676,980.

#### 6. Triabodies

Triabodies are also within the scope of the invention. Such antibodies are described for instance in Iliades et al., FEBS Letters, 409:437-441 (1997) and Korrt et al., Protein Engineering, 10:423-433 (1997).

#### 7. Other Modifications

Other modifications of the Apo-2 antibodies are contemplated. For example, it may be desirable to modify the antibodies of the invention with respect to effector function, so as to enhance the therapeutic effectiveness of the antibodies. For instance, cysteine residue(s) may be introduced into the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing [see, e.g., Caron et al., J. Exp. Med., 176:1191-1195 (1992); Shopes, J. Immunol., 148:2918-2922 (1992). Homodimeric antibodies may also be prepared using heterobifunctional cross-linkers as described in Wolff et al., Cancer Research, 53:2560-2565 (1993). Ghetie et al., Proc. Natl. Acad. Sci., 94:7509-7514 (1997), further describe preparation of IgG-IgG homodimers and disclose that such homodimers can enhance apoptotic activity as compared to the monomers. Alternatively, the antibodies can be engineered to have dual Fc regions [see, Stevenson et al., Anti-Cancer Drug Design, 3:219-230 (1989)].

It may be desirable to modify the amino acid sequences of the antibodies disclosed herein. Sequences within the scFv complementary determining or linker regions (as shown in Figure 16) may be modified for instance to modulate the biological activities of these antibodies. Variations in the full-length scFv sequence or in various domains of the scFv molecules described herein, can be



made, for example, using any of the techniques and guidelines for conservative and non-conservative mutations set forth, for instance, in U.S. Pat. No. 5,364,934. Variations may be a substitution, deletion or insertion of one or more codons encoding a scFv that results in a change in the amino acid sequence of the scFv as compared with the native sequence scFv. Optionally, the variation is by substitution of at least one amino acid with any other amino acid in one or more of the domains of the scFv molecule. The variations can be made using methods known in the art such as oligonucleotide-mediated (site-directed) mutagenesis, alanine scanning, and PCR mutagenesis. Site-directed mutagenesis [Carter et al., Nucl. Acids Res., 13:4331 (1986); Zoller et al., Nucl. Acids Res., 10:6487 (1987)], cassette mutagenesis [Wells et al., Gene, 34:315 (1985)], restriction selection mutagenesis [Wells et al., Philos. Trans. R. Soc. London SerA, 317:415 (1986)] or other known techniques can be performed on the cloned DNA to produce the scFv variant DNA.

The antibodies may optionally be covalently attached or conjugated to one or more chemical groups. A polyol, for example, can be conjugated to an antibody molecule at one or more amino acid residues, including lysine residues as disclosed in WO 93/00109. Optionally, the polyol is a poly(alkylene glycol), such as poly(ethylene glycol) (PEG), however, those skilled in the art recognize that other polyols, such as, for example, poly(propylene glycol) and polyethylene-polypropylene glycol copolymers, can be employed using techniques for conjugating PEG to polypeptides. A variety of methods for pegylating polypeptides have been described. See, e.g. U.S. Patent No. 4,179,337 which discloses the conjugation of a number of hormones and enzymes to PEG and polypropylene glycol to produce physiologically active compositions having reduced immunogenicities.

The antibodies may also be fused or linked to another heterologous polypeptide or amino acid sequence such as an epitope tag. Epitope tag polypeptides and methods of their use are described above in Section A, paragraph 8. Any of the tags described herein may be linked to the antibodies. The Examples below, for instance, describe His-tagged and gD-tagged single-chain antibodies.

#### D. Therapeutic Uses for Apo-2 Antibodies

The Apo-2 antibodies of the invention have therapeutic utility. Agonistic Apo-2 antibodies, for instance, may be employed to activate or stimulate apoptosis in cancer cells. Accordingly, the invention provides methods for treating cancer using such Apo-2 antibodies. It is of course contemplated that the methods of the invention can be employed in combination with still other therapeutic techniques such as surgery.

The agonist is preferably administered to the mammal in a carrier. Suitable carriers and their formulations are described in Remington's Pharmaceutical Sciences, 16th ed., 1980, Mack Publishing Co., edited by Oslo et al. Typically, an appropriate amount of a pharmaceutically-acceptable salt is used in the formulation to render the formulation isotonic. Examples of a pharmaceutically-acceptable carrier include saline, Ringer's solution and dextrose solution. The pH of the solution is preferably from about 5 to about 8, and more preferably from about 7 to about 7.5. Further carriers include sustained release preparations such as semipermeable matrices of solid hydrophobic polymers containing the agonist, which matrices are in the form of shaped articles, e.g., films, liposomes or microparticles. It will be apparent to those persons skilled in the art that certain carriers may be more preferable depending upon, for instance, the route of administration and concentration of agonist being administered.

The agonist antibody can be administered to the mammal by injection (e.g., intravenous, intraperitoneal, subcutaneous, intramuscular), or by other methods such as infusion that ensure its delivery to the bloodstream in an effective form. The agonist may also be administered by intratumoral, peritumoral, intralesional, or perilesional routes, to exert local as well as systemic therapeutic effects. Local or intravenous injection is preferred.

Effective dosages and schedules for administering the agonist antibody may be determined empirically, and making such determinations is within the skill in the art. Those skilled in the art will understand that the dosage of agonist that must be administered will vary depending on, for example, the mammal which will receive the agonist, the route of administration, the

particular type of agonist used and other drugs being administered to the mammal. Guidance in selecting appropriate doses for antibody agonists is found in the literature on therapeutic uses of antibodies, e.g., Handbook of Monoclonal Antibodies, Ferrone et al., eds., Noyes Publications, Park Ridge, N.J., (1985) ch. 22 and pp. 303-357; Smith et al., Antibodies in Human Diagnosis and Therapy, Haber et al., eds., Raven Press, New York (1977) pp. 365-389. A typical daily dosage of the agonist used alone might range from about 1 µg/kg to up to 100 mg/kg of body weight or more per day, depending on the factors mentioned above.

The agonist antibody may also be administered to the mammal in combination with effective amounts of one or more other therapeutic agents or in conjunction with radiation treatment. Therapeutic agents contemplated include chemotherapeutics as well as immunoadjuvants and cytokines. Chemotherapies contemplated by the invention include chemical substances or drugs which are known in the art and are commercially available, such as Doxorubicin, 5-Fluorouracil, Cytosine arabinoside ("Ara-C"), Cyclophosphamide, Thiotepa, Busulfan, Cytosine, Taxol, Methotrexate, Cisplatin, Melphalan, Vinblastine and Carboplatin. The agonist may be administered sequentially or concurrently with the one or more other therapeutic agents. The amounts of agonist and therapeutic agent depend, for example, on what type of drugs are used, the cancer being treated, and the scheduling and routes of administration but would generally be less than if each were used individually.

Following administration of agonist to the mammal, the mammal's cancer and physiological condition can be monitored in various ways well known to the skilled practitioner. For instance, tumor mass may be observed physically or by standard x-ray imaging techniques.

The Apo-2 antibodies of the invention may also be useful in enhancing immune-mediated cell death in cells expressing Apo-2, for instance, through complement fixation or ADCC. Alternatively, antagonistic antibodies may be used to block excessive apoptosis (for instance in neurodegenerative disease) or to block potential autoimmune/inflammatory effects of Apo-2 resulting from NF-κB

activation. Such antagonistic antibodies can be utilized according to the therapeutic methods and techniques described above.

E. Non-therapeutic Uses for Apo-2 Antibodies

Apo-2 antibodies may further be used in diagnostic assays for Apo-2, e.g., detecting its expression in specific cells, tissues, or serum. Various diagnostic assay techniques known in the art may be used, such as competitive binding assays, direct or indirect sandwich assays and immunoprecipitation assays conducted in either heterogeneous or homogeneous phases [Zola, Monoclonal Antibodies: A Manual of Techniques, CRC Press, Inc. (1987) pp. 147-158]. The antibodies used in the diagnostic assays can be labeled with a detectable moiety. The detectable moiety should be capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety may be a radioisotope, such as  $^3\text{H}$ ,  $^{14}\text{C}$ ,  $^{32}\text{P}$ ,  $^{35}\text{S}$ , or  $^{125}\text{I}$ , a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin, or an enzyme, such as alkaline phosphatase, beta-galactosidase or horseradish peroxidase. Any method known in the art for conjugating the antibody to the detectable moiety may be employed, including those methods described by Hunter et al., Nature, 144:945 (1962); David et al., Biochemistry, 13:1014 (1974); Pain et al., J. Immunol. Meth., 40:219 (1981); and Nygren, J. Histochem. and Cytochem., 30:407 (1982).

Apo-2 antibodies also are useful for the affinity purification of Apo-2 from recombinant cell culture or natural sources. In this process, the antibodies against Apo-2 are immobilized on a suitable support, such as Sephadex resin or filter paper, using methods well known in the art. The immobilized antibody then is contacted with a sample containing the Apo-2 to be purified, and thereafter the support is washed with a suitable solvent that will remove substantially all the material in the sample except the Apo-2, which is bound to the immobilized antibody. Finally, the support is washed with another suitable solvent that will release the Apo-2 from the antibody.

F. Kits Containing Apo-2 or Apo-2 Antibodies

In a further embodiment of the invention, there are provided articles of manufacture and kits containing Apo-2 or Apo-2 antibodies which can be used, for instance, for the therapeutic or

non-therapeutic applications described above. The article of manufacture comprises a container with a label. Suitable containers include, for example, bottles, vials, and test tubes. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which includes an active agent that is effective for therapeutic or non-therapeutic applications, such as described above. The active agent in the composition is Apo-2 or an Apo-2 antibody. The label on the container indicates that the composition is used for a specific therapy or non-therapeutic application, and may also indicate directions for either *in vivo* or *in vitro* use, such as those described above.

The kit of the invention will typically comprise the container described above and one or more other containers comprising materials desirable from a commercial and user standpoint, including buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

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The following examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

All patent and literature references cited in the present specification are hereby incorporated by reference in their entirety.

25

#### EXAMPLES

All restriction enzymes referred to in the examples were purchased from New England Biolabs and used according to manufacturer's instructions. All other commercially available reagents referred to in the examples were used according to manufacturer's instructions unless otherwise indicated. The source of those cells identified in the following examples, and throughout the specification, by ATCC accession numbers is the American Type Culture Collection, Manassas, VA.

35

#### EXAMPLE 1

##### Isolation of cDNA clones Encoding Human Apo-2

Expressed sequence tag (EST) DNA databases (LIFESEQ™, Incyte Pharmaceuticals, Palo Alto, CA) were searched and an EST was

identified which showed homology to the death domain of the Apo-3 receptor [Marsters et al., Curr. Biol., 6:750 (1996)]. Human pancreas and kidney lgt10 bacteriophage cDNA libraries (both purchased from Clontech) were ligated into pRK5 vectors as follows.

- 5 Reagents were added together and incubated at 16°C for 16 hours: 5X T4 ligase buffer (3 ml); pRK5, Xho1, Not1 digested vector, 0.5 mg, 1 ml); cDNA (5 ml) and distilled water (6 ml). Subsequently, additional distilled water (70 ml) and 10 mg/ml tRNA (0.1 ml) were added and the entire reaction was extracted through
- 10 phenol:chloroform:isoamyl alcohol (25:24:1). The aqueous phase was removed, collected and diluted into 5M NaCl (10 ml) and absolute ethanol (-20°C, 250 ml). This was then centrifuged for 20 minutes at 14,000 x g, decanted, and the pellet resuspended into 70% ethanol (0.5 ml) and centrifuged again for 2 minutes at 14,000 x g.
- 15 The DNA pellet was then dried in a speedvac and eluted into distilled water (3 ml) for use in the subsequent procedure.

- The ligated cDNA/pRK5 vector DNA prepared previously was chilled on ice to which was added electrocompetent DH10B bacteria (Life Tech., 20 ml). The bacteria vector mixture was then
- 20 electroporated as per the manufacturers recommendation. Subsequently SOC media (1 ml) was added and the mixture was incubated at 37°C for 30 minutes. The transformants were then plated onto 20 standard 150 mm LB plates containing ampicillin and incubated for 16 hours (37°C) to allow the colonies to grow.
- 25 Positive colonies were then scraped off and the DNA isolated from the bacterial pellet using standard CsCl-gradient protocols.

- An enriched 5'-cDNA library was then constructed to obtain a bias of cDNA fragments which preferentially represents the 5' ends of cDNA's contained within the library. 10 mg of the
- 30 pooled isolated full-length library plasmid DNA (41 ml) was combined with Not 1 restriction buffer (New England Biolabs, 5 ml) and Not 1 (New England Biolabs, 4 ml) and incubated at 37°C for one hour. The reaction was extracted through phenol:chloroform:isoamyl alcohol (25:24:1, 50 ml), the aqueous phase removed, collected and
- 35 resuspended into 5M NaCl (5 ml) and absolute ethanol (-20°C, 150 ml). This was then centrifuged for 20 minutes at 14,000 x g, decanted, resuspended into 70% ethanol (0.5 ml) and centrifuged again for 2 minutes at 14,000 x g. The supernatant was then

removed, the pellet dried in a speedvac and resuspended in distilled water (10 ml).

The following reagents were brought together and incubated at 37°C for 2 hours: distilled water (3 ml); linearized  
5 DNA library (1 mg, 1 ml); Ribonucleotide mix (Invitrogen, 10 ml);  
transcription buffer (Invitrogen, 2 ml) and Sp6 enzyme mix. The  
reaction was then extracted through phenol:chloroform:isoamyl  
alcohol (25:24:1, 50 ml) and the aqueous phase was removed,  
collected and resuspended into 5M NaCl (5 ml) and absolute ethanol  
10 (-20°C, 150 ml) and centrifuged for 20 minutes at 14,000 x g. The  
pellet was then decanted and resuspended in 70% ethanol (0.5 ml),  
centrifuged again for 2 minutes at 14,000 x g, decanted, dried in a  
speedvac and resuspended into distilled water (10 ml).

The following reagents were added together and incubated  
15 at 16°C for 16 hours: 5X T4 ligase buffer (Life Tech., 3 ml); pRK5  
Cla-Sal digested vector, 0.5 mg, 1 ml); cDNA (5 ml); distilled  
water (6 ml). Subsequently, additional distilled water (70 ml) and  
10 mg/ml tRNA (0.1 ml) was added and the entire reaction was  
extracted through phenol:chloroform:isoamyl alcohol (25:24:1, 100  
20 ml). The aqueous phase was removed, collected and diluted by 5M  
NaCl (10 ml) and absolute ethanol (-20°C, 250 ml) and centrifuged  
for 20 minutes at 14,000 x g. The DNA pellet was decanted,  
resuspended into 70% ethanol (0.5 ml) and centrifuged again for 2  
minutes at 14,000 x g. The supernatant was removed and the residue  
25 pellet was dried in a speedvac and resuspended in distilled water  
(3 ml). The ligated cDNA/pSST-amy.1 vector DNA was chilled on ice  
to which was added electrocompetent DH10B bacteria (Life Tech., 20  
ml). The bacteria vector mixture was then electroporated as  
recommended by the manufacturer. Subsequently, SOC media (Life  
30 Tech., 1 ml) was added and the mixture was incubated at 37°C for 30  
minutes. The transformants were then plated onto 20 standard 150  
mm LB plates containing ampicillin and incubated for 16 hours  
(37°C). Positive colonies were scraped off the plates and the DNA  
was isolated from the bacterial pellet using standard protocols,  
35 e.g. CsCl-gradient.

The cDNA libraries were screened by hybridization with a  
synthetic oligonucleotide probe:

GGGAGCCGCTCATGAGGAAGTTGGGCCTCATGGACAATGAGATAAAGGTGGCTAAAGCTGAGGCAGC  
GGG (SEQ ID NO:3) based on the EST.

Three cDNA clones were sequenced in entirety. The overlapping coding regions of the cDNAs were identical except for  
5 codon 410 (using the numbering system for Fig. 1); this position encoded a leucine residue (TTG) in both pancreatic cDNAs, and a methionine residue (ATG) in the kidney cDNA, possibly due to polymorphism.

The entire nucleotide sequence of Apo-2 is shown in  
10 Figure 1 (SEQ ID NO:2). Clone 27868 (also referred to as pRK5-Apo-2 deposited as ATCC 209021, as indicated below) contains a single open reading frame with an apparent translational initiation site at nucleotide positions 140-142 [Kozak et al., supra] and ending at the stop codon found at nucleotide positions 1373-1375 (Fig. 1; SEQ  
15 ID NO:2). The predicted polypeptide precursor is 411 amino acids long, a type I transmembrane protein, and has a calculated molecular weight of approximately 45 kDa. Hydropathy analysis (not shown) suggested the presence of a signal sequence (residues 1-53), followed by an extracellular domain (residues 54-182), a  
20 transmembrane domain (residues 183-208), and an intracellular domain (residues 209-411) (Fig. 2A; SEQ ID NO:1). N-terminal amino acid sequence analysis of Apo-2-IgG expressed in 293 cells showed that the mature polypeptide starts at amino acid residue 54, indicating that the actual signal sequence comprises residues 1-53.  
25 Apo-2 polypeptide is obtained or obtainable by expressing the molecule encoded by the cDNA insert of the deposited ATCC 209021 vector.

TNF receptor family proteins are typically characterized by the presence of multiple (usually four) cysteine-rich domains in  
30 their extracellular regions -- each cysteine-rich domain being approximately 45 amino acids long and containing approximately 6, regularly spaced, cysteine residues. Based on the crystal structure of the type 1 TNF receptor, the cysteines in each domain typically form three disulfide bonds in which usually cysteines 1  
35 and 2, 3 and 5, and 4 and 6 are paired together. Like DR4, Apo-2 contains two extracellular cysteine-rich pseudorepeats (Fig. 2A), whereas other identified mammalian TNFR family members contain three or more such domains [Smith et al., Cell, 76:959 (1994)].



The cytoplasmic region of Apo-2 contains a death domain (amino acid residues 324-391 shown in Fig. 1; see also Fig. 2A) which shows significantly more amino acid sequence identity to the death domain of DR4 (64%) than to the death domain of TNFR1 (30%); CD95 (19%); or Apo-3/DR3 (29%) (Fig. 2B). Four out of six death domain amino acids that are required for signaling by TNFR1 [Tartaglia et al., supra] are conserved in Apo-2 while the other two residues are semi-conserved (see Fig. 2B).

Based on an alignment analysis (using the ALIGN<sup>TM</sup> computer program) of the full-length sequence, Apo-2 shows more sequence identity to DR4 (55%) than to other apoptosis-linked receptors, such as TNFR1 (19%); CD95 (17%); or Apo-3 (also referred to as DR3, WSL-1 or TRAMP) (29%).

## 15 EXAMPLE 2

### A. Expression of Apo-2 ECD

A soluble extracellular domain (ECD) fusion construct was prepared. An Apo-2 ECD (amino acid residues 1-184 shown in Figure 1) was obtained by PCR and fused to a C-terminal Flag epitope tag (Sigma). (The Apo-2 ECD construct included residues 183 and 184 shown in Figure 1 to provide flexibility at the junction, even though residues 183 and 184 are predicted to be in the transmembrane region). The Flag epitope-tagged molecule was then inserted into pRK5, and expressed by transient transfection into human 293 cells (ATCC CRL 1573).

After a 48 hour incubation, the cell supernatants were collected and either used directly for co-precipitation studies (see Example 3) or subjected to purification of the Apo-2 ECD-Flag by affinity chromatography on anti-Flag agarose beads, according to manufacturer's instructions (Sigma).

### B. Expression of Apo-2 ECD as an Immunoadhesin

A soluble Apo-2 ECD immunoadhesin construct was prepared. The Apo-2 ECD (amino acids 1-184 shown in Fig. 1) was fused to the hinge and Fc region of human immunoglobulin G<sub>1</sub> heavy chain in pRK5 as described previously [Ashkenazi et al., Proc. Natl. Acad. Sci., 88:10535-10539 (1991)]. The immunoadhesin was expressed by transient transfection into human 293 cells and

purified from cell supernatants by protein A affinity chromatography, as described by Ashkenazi et al., supra.

### EXAMPLE 3

#### 5           Immunoprecipitation Assay Showing Binding Interaction           Between Apo-2 and Apo-2 Ligand

To determine whether Apo-2 and Apo-2L interact or associate with each other, supernatants from mock-transfected 293 cells or from 293 cells transfected with Apo-2 ECD-Flag (described  
10 in Example 2 above) (5 ml) were incubated with 5 µg poly-histidine-tagged soluble Apo-2L [Pitti et al., supra] for 30 minutes at room temperature and then analyzed for complex formation by a co-precipitation assay.

The samples were subjected to immunoprecipitation using  
15 25 µl anti-Flag conjugated agarose beads (Sigma) or Nickel-conjugated agarose beads (Qiagen). After a 1.5 hour incubation at 4° C, the beads were spun down and washed four times in phosphate buffered saline (PBS). By using anti-Flag agarose, the Apo-2L was precipitated through the Flag-tagged Apo-2 ECD; by using Nickel-  
20 agarose, the Apo-2 ECD was precipitated through the His-tagged Apo-2L. The precipitated proteins were released by boiling the beads for 5 minutes in SDS-PAGE buffer, resolved by electrophoresis on 12% polyacrylamide gels, and then detected by immunoblot with anti-Apo-2L or anti-Flag antibody (2 µg/ml) as described in Marsters et  
25 al., J. Biol. Chem., (1997).

The results, shown in Figure 3, indicate that the Apo-2 ECD and Apo-2L can associate with each other.

The binding interaction was further analyzed by purifying Apo-2 ECD from the transfected 293 cell supernatants with  
30 anti-Flag beads (see Example 2) and then analyzing the samples on a BIAcore™ instrument. The BIAcore™ analysis indicated a dissociation constant ( $K_d$ ) of about 1 nM. BIAcore™ analysis also showed that the Apo-2 ECD is not capable of binding other apoptosis-inducing TNF family members, namely, TNF-alpha  
35 (Genentech, Inc., Pennica et al., Nature, 312:712 (1984), lymphotoxin-alpha (Genentech, Inc.), or Fas/Apo-1 ligand (Alexis Biochemicals). The data thus shows that Apo-2 is a specific receptor for Apo-2L.

EXAMPLE 4Induction of Apoptosis by Apo-2

Because death domains can function as oligomerization  
5 interfaces, over-expression of receptors that contain death domains  
may lead to activation of signaling in the absence of ligand  
[Frazer et al., supra, Nagata et al., supra]. To determine whether  
Apo-2 was capable of inducing cell death, human 293 cells or HeLa  
cells (ATCC CCL 2.2) were transiently transfected by calcium  
10 phosphate precipitation (293 cells) or electroporation (HeLa cells)  
with a pRK5 vector or pRK5-based plasmids encoding Apo-2 and/or  
CrmA. When applicable, the total amount of plasmid DNA was  
adjusted by adding vector DNA. Apoptosis was assessed 24 hours  
after transfection by morphology (Fig. 4A); DNA fragmentation (Fig.  
15 4B); or by FACS analysis of phosphatidylserine exposure (Fig. 4C)  
as described in Marsters et al., Curr. Biol., 6:1669 (1996). As  
shown in Figs. 4A and 4B, the Apo-2 transfected 293 cells underwent  
marked apoptosis.

For samples assayed by FACS, the HeLa cells were co-  
20 transfected with pRK5-CD4 as a marker for transfection and  
apoptosis was determined in CD4-expressing cells; FADD was co-  
transfected with the Apo-2 plasmid; the data are means  $\pm$  SEM of at  
least three experiments, as described in Marsters et al., Curr.  
Biol., 6:1669 (1996). The caspase inhibitors, DEVD-fmk (Enzyme  
25 Systems) or z-VAD-fmk (Research Biochemicals Intl.) were added at  
200  $\mu$ M at the time of transfection. As shown in Fig. 4C, the  
caspase inhibitors CrmA, DEVD-fmk, and z-VAD-fmk blocked apoptosis  
induction by Apo-2, indicating the involvement of Ced-3-like  
proteases in this response.

30 FADD is an adaptor protein that mediates apoptosis  
activation by CD95, TNFR1, and Apo-3/DR3 [Nagata et al., supra],  
but does not appear necessary for apoptosis induction by Apo-2L  
[Marsters et al., supra] or by DR4 [Pan et al., supra]. A  
dominant-negative mutant form of FADD, which blocks apoptosis  
35 induction by CD95, TNFR1, or Apo-3/DR3 [Frazer et al., supra;  
Nagata et al., supra; Chinnayian et al., supra] did not inhibit  
apoptosis induction by Apo-2 when co-transfected into HeLa cells  
with Apo-2 (Fig. 4C). These results suggest that Apo-2 signals  
apoptosis independently of FADD. Consistent with this conclusion,

a glutathione-S-transferase fusion protein containing the Apo-2 cytoplasmic region did not bind to *in vitro* transcribed and translated FADD (data not shown).

5

#### EXAMPLE 5

##### Inhibition of Apo-2L Activity by Soluble Apo-2 ECD

Soluble Apo-2L (0.5 µg/ml, prepared as described in Pitti et al., supra) was pre-incubated for 1 hour at room temperature with PBS buffer or affinity-purified Apo-2 ECD (5  
10 µg/ml) together with anti-Flag antibody (Sigma) (1 µg/ml) and added to HeLa cells. After a 5 hour incubation, the cells were analyzed for apoptosis by FACS (as above) (Fig. 4D).

Apo-2L induced marked apoptosis in HeLa cells, and the soluble Apo-2 ECD was capable of blocking Apo-2L action (Fig. 4D),  
15 confirming a specific interaction between Apo-2L and Apo-2. Similar results were obtained with the Apo-2 ECD immunoadhesin (Fig. 4D). Dose-response analysis showed half-maximal inhibition at approximately 0.3 nM Apo-2 immunoadhesin (Fig. 4E).

20

#### EXAMPLE 6

##### Activation of NF-κB by Apo-2

An assay was conducted to determine whether Apo-2 activates NF-κB.

HeLa cells were transfected with pRK5 expression  
25 plasmids encoding full-length native sequence Apo-2, DR4 or Apo-3 and harvested 24 hours after transfection. Nuclear extracts were prepared and 1 µg of nuclear protein was reacted with a <sup>32</sup>P-labelled NF-κB-specific synthetic oligonucleotide probe

ATCAGGGACTTTCCGCTGGGGACTTTCCG (SEQ ID NO:4) [see, also, MacKay et  
30 al., J. Immunol., 153:5274-5284 (1994)], alone or together with a 50-fold excess of unlabelled probe, or with an irrelevant <sup>32</sup>P-labelled synthetic oligonucleotide

AGGATGGGAAGTGTGTGATATATCCTTGAT (SEQ ID NO:5). In some samples,  
antibody to p65/RelA subunits of NF-κB (1 µg/ml; Santa Cruz  
35 Biotechnology) was added. DNA binding was analyzed by an electrophoretic mobility shift assay as described by Hsu et al., supra; Marsters et al., supra, and MacKay et al., supra.

The results are shown in Fig. 5. As shown in Fig. 5A, upon transfection into HeLa cells, both Apo-2 and DR4 induced significant NF- $\kappa$ B activation as measured by the electrophoretic mobility shift assay; the level of activation was comparable to activation observed for Apo-3/DR3. Antibody to the p65/RelA subunit of NF- $\kappa$ B inhibited the mobility of the NF- $\kappa$ B probe, implicating p65 in the response to all 3 receptors.

An assay was also conducted to determine if Apo-2L itself can regulate NF- $\kappa$ B activity. HeLa cells or MCF7 cells (human breast adenocarcinoma cell line, ATCC HTB 22) were treated with PBS buffer, soluble Apo-2L (Pitti et al., *supra*) or TNF-alpha (Genentech, Inc., see Pennica et al., *Nature*, 312:721 (1984)) (1  $\mu$ g/ml) and assayed for NF- $\kappa$ B activity as above. The results are shown in Fig. 5B. The Apo-2L induced a significant NF- $\kappa$ B activation in the treated HeLa cells but not in the treated MCF7 cells; the TNF-alpha induced a more pronounced activation in both cell lines. Several studies have disclosed that NF- $\kappa$ B activation by TNF can protect cells against TNF-induced apoptosis [Nagata, *supra*].

The effects of a NF- $\kappa$ B inhibitor, ALLN (N-acetyl-Leu-Leu-norleucinal) and a transcription inhibitor, cyclohexamide, were also tested. The HeLa cells (plated in 6-well dishes) were preincubated with PBS buffer, ALLN (Calbiochem) (40  $\mu$ g/ml) or cyclohexamide (Sigma) (50  $\mu$ g/ml) for 1 hour before addition of Apo-2L (1  $\mu$ g/ml). After a 5 hour incubation, apoptosis was analyzed by FACS (see Fig. 5C).

The results are shown in Fig. 5C. Both ALLN and cyclohexamide increased the level of Apo-2L-induced apoptosis in the HeLa cells. The data indicates that Apo-2L can induce protective NF- $\kappa$ B-dependent genes. The data also indicates that Apo-2L is capable of activating NF- $\kappa$ B in certain cell lines and that both Apo-2 and DR4 may mediate that function.

#### EXAMPLE 7

##### Expression of Apo-2 in Mammalian Tissues

##### A. Northern Blot Analysis

Expression of Apo-2 mRNA in human tissues was examined by Northern blot analysis. Human RNA blots were hybridized to a

4.6 kilobase <sup>32</sup>P-labelled DNA probe based on the full length Apo-2 cDNA; the probe was generated by digesting the pRK5-Apo-2 plasmid with EcoRI. Human fetal RNA blot MTN (Clontech), human adult RNA blot MTN-II (Clontech), and human cancer cell line RNA blot (Clontech) were incubated with the DNA probes. Blots were incubated with the probes in hybridization buffer (5X SSPE; 2X Denhardt's solution; 100 mg/mL denatured sheared salmon sperm DNA; 50% formamide; 2% SDS) for 60 hours at 42°C. The blots were washed several times in 2X SSC; 0.05% SDS for 1 hour at room temperature, followed by a 30 minute wash in 0.1X SSC; 0.1% SDS at 50°C. The blots were developed after overnight exposure.

As shown in Fig. 6A, a predominant mRNA transcript of approximately 4.6kb was detected in multiple tissues. Expression was relatively high in fetal and adult liver and lung, and in adult ovary and peripheral blood leukocytes (PBL), while no mRNA expression was detected in fetal and adult brain. Intermediate levels of expression were seen in adult colon, small intestine, testis, prostate, thymus, pancreas, kidney, skeletal muscle, placenta, and heart. Several adult tissues that express Apo-2, e.g., PBL, ovary, and spleen, have been shown previously to express DR4 [Pan et al., *supra*], however, the relative levels of expression of each receptor mRNA appear to be different.

As shown in Fig. 6B, Apo-2 mRNA was expressed relatively high in 6 of 8 human cancer cell lines examined, namely, HL60 promyelocytic leukemia, HeLa S3 cervical carcinoma, K562 chronic myelogenous leukemia, SW 480 colorectal adenocarcinoma, A549 lung carcinoma, and G361 melanoma. There was also detectable expression in Burkitt's lymphoma (Raji) cells. Thus, Apo-2 may be useful as a target for inducing apoptosis in cancer cells from lymphoid as well as non-lymphoid tumors.

#### B. In Situ Hybridization

Expression of Apo-2 in normal and in cancerous human tissues was examined by *in situ* hybridization. In addition, several different chimp and rhesus monkey tissues were examined for Apo-2 expression. These tissues included: human fetal tissues (E12-E16 weeks) - placenta, umbilical cord, liver, kidney, adrenal gland, thyroid, lung, heart, great vessels, esophagus, stomach, small intestine, spleen, thymus, pancreas, brain, eye, spinal cord,

body wall, pelvis and lower limb; adult human tissues - kidney, bladder, adrenal gland, spleen, lymph node, pancreas, lung, skin, retina, liver; chimp tissues - salivary gland, stomach, thyroid, parathyroid, tongue, thymus, ovary, lymph node, and peripheral nerve; rhesus monkey tissues - cerebral cortex, hippocampus, cerebellum and penis; human tumor tissue - lung adenocarcinoma, testis, lung carcinoma, breast carcinoma, fibroadenoma, soft tissue sarcoma.

Tissue samples were paraffin-embedded and sectioned. Later, the sectioned tissues were deparaffinized and the slides placed in water. The slides were rinsed twice for five minutes at room temperature in 2X SSC. After rinsing, the slides were placed in 20 µg/ml proteinase K (in RNase-free buffer) for 15 minutes at 37 °C (for fetal tissues) or 8X proteinase K for 30 minutes at 37 °C (for formalin tissues). The slides were then rinsed again in 0.5X SSC and dehydrated. Prior to hybridization, the slides were placed in a plastic box lined with buffer (4X SSC, 50% formamide)-saturated filter paper. The tissues were covered with 50 µl hybridization buffer (3.75g Dextran sulfate plus 6 ml water; vortexed and heated for 2 minutes; cooled on ice and 18.75 ml formamide, 3.75 ml 20X SSC and 9 ml water added) and incubated at 42 °C for 1 to 4 hours.

Hybridization was conducted using a <sup>33</sup>P-labelled probe consisting of nucleotides 706-1259 of SEQ ID NO:2. The probe was added to the slides in hybridization buffer and incubated overnight at 55 °C. Multiple washing steps were then performed sequentially as follows: twice for 10 minutes at room temperature in 2X SSC, EDTA buffer (400 ml 20X SSC, 16 ml 0.25M EDTA); once for 30 minutes at 37 °C in 20 µg/ml RNase A; twice for 10 minutes at room temperature in 2X SSC, EDTA buffer; once for 2 hours at 55 °C in 0.1X SSC, EDTA buffer; twice for 10 minutes at room temperature in 0.5X SSC. Dehydration was performed for 2 minutes each in 50%, 70%, 90% EtOH containing 0.3 M NH<sub>4</sub>AC. Finally, the slides were air-dried for 2 hours and exposed to film.

Expression of Apo-2 in the fetal tissues appeared strongest over hepatocytes in liver, developing glomeruli in kidney, adrenal cortex, and epithelium of gastrointestinal tract. Moderate expression was observed over epithelial cells in lung and

at sites of vascularization of a bone growth plate. A relatively low level expression was observed over thyroid epithelial cells and cells in cardiac ventricles. Expression was observed over lymphoid cells in the thymic medulla, developing lymph glands and placenta  
5 cytotrophoblast cells.

Expression of Apo-2 in adult tissues was observed over resting oocytes in primordial follicles and low levels over granulosa cells of developing follicles in chimp ovary. Expression was observed in cirrhotic livers over hepatocytes at the edge of  
10 nodules (i.e., area of damage, normal adult liver was negative). Other tissues were negative for expression.

In the cancer tissues examined, Apo-2 expression was found in two lung adenocarcinomas and two germ cell tumors of the testis. Two additional lung carcinomas (one squamous) were  
15 negative. One of five breast carcinomas was positive (there was expression in normal breast tissue). In a fibroadenoma, there appeared to be expression over both epithelial and stromal elements. A soft tissue sarcoma was also positive. Other tissues examined were negative.

20

#### EXAMPLE 8

##### Chromosomal Localization of the Apo-2 gene

Chromosomal localization of the human Apo-2 gene was examined by radiation hybrid (RH) panel analysis. RH mapping was  
25 performed by PCR using a human-mouse cell radiation hybrid panel (Research Genetics) and primers based on the coding region of the Apo-2 cDNA [Gelb et al., Hum. Genet., 98:141 (1996)]. Analysis of the PCR data using the Stanford Human Genome Center Database indicates that Apo-2 is linked to the marker D8S481, with an LOD of  
30 11.05; D8S481 is linked in turn to D8S2055, which maps to human chromosome 8p21. A similar analysis of DR4 showed that DR4 is linked to the marker D8S2127 (with an LOD of 13.00), which maps also to human chromosome 8p21.

To Applicants' present knowledge, to date, no other  
35 member of the TNFR gene family has been located to chromosome 8.



EXAMPLE 9Preparation of Monoclonal Antibodies Specific for Apo-2

Balb/c mice (obtained from Charles River Laboratories) were immunized by injecting 0.5µg/50µl of an Apo-2 ECD immunoadhesin protein (diluted in MPL-TDM adjuvant purchased from Ribi Immunochemical Research Inc., Hamilton, MT) 11 times into each hind foot pad at 3-4 day intervals. The Apo-2 ECD immunoadhesin protein was generated by fusing an extracellular domain sequence of Apo-2 (amino acids 1-184 shown in Fig. 1) to the hinge and Fc region of human immunoglobulin G<sub>1</sub> heavy chain in pRK5 as described previously [Ashkenazi et al., Proc. Natl. Acad. Sci., 88:10535-10539 (1991)]. The immunoadhesin protein was expressed by transient transfection into human 293 cells and purified from cell supernatants by protein A affinity chromatography, as described by Ashkenazi et al., supra (See also Example 2B above).

Three days after the final boost, popliteal lymph nodes were removed from the mice and a single cell suspension was prepared in DMEM media (obtained from Biowhitakker Corp.) supplemented with 1% penicillin-streptomycin. The lymph node cells were then fused with murine myeloma cells P3X63AgU.1 (ATCC CRL 1597) using 35% polyethylene glycol and cultured in 96-well culture plates. Hybridomas resulting from the fusion were selected in HAT medium. Ten days after the fusion, hybridoma culture supernatants were screened in an ELISA to test for the presence of monoclonal antibodies binding to the Apo-2 ECD immunoadhesin protein.

In the ELISA, 96-well microtiter plates (Maxisorb; Nunc, Kamstrup, Denmark) were coated by adding 50 µl of 2 µg/ml goat anti-human IgG Fc (purchased from Cappel Laboratories) in PBS to each well and incubating at 4°C overnight. The plates were then washed three times with wash buffer (PBS containing 0.05% Tween 20). The wells in the microtiter plates were then blocked with 50 µl of 2.0% bovine serum albumin in PBS and incubated at room temperature for 1 hour. The plates were then washed again three times with wash buffer.

After the washing step, 50 µl of 0.4 µg/ml Apo-2 ECD immunoadhesin protein (as described above) in assay buffer was added to each well. The plates were incubated for 1 hour at room

temperature on a shaker apparatus, followed by washing three times with wash buffer.

Following the wash steps, 100  $\mu$ l of the hybridoma supernatants or purified antibody (using Protein A-sepharose columns) (1  $\mu$ g/ml) was added to designated wells in the presence of CD4-IgG. 100  $\mu$ l of P3X63AgU.1 myeloma cell conditioned medium was added to other designated wells as controls. The plates were incubated at room temperature for 1 hour on a shaker apparatus and then washed three times with wash buffer.

Next, 50  $\mu$ l HRP-conjugated goat anti-mouse IgG Fc (purchased from Cappel Laboratories), diluted 1:1000 in assay buffer (0.5% bovine serum albumin, 0.05% Tween-20, 0.01% Thimersol in PBS), was added to each well and the plates incubated for 1 hour at room temperature on a shaker apparatus. The plates were washed three times with wash buffer, followed by addition of 50  $\mu$ l of substrate (TMB microwell peroxidase substrate, Kirkegaard & Perry, Gaithersburg, MD) to each well and incubation at room temperature for 10 minutes. The reaction was stopped by adding 50  $\mu$ l of TMB 1-component stop solution (diethyl glycol, Kirkegaard & Perry) to each well, and absorbance at 450 nm was read in an automated microtiter plate reader.

Of the hybridoma supernatants screened in the ELISA, 22 supernatants tested positive (calculated as approximately 4 times above background). The supernatants testing positive in the ELISA were further analyzed by FACS analysis using 9D cells (a human B lymphoid cell line expressing Apo-2; Genentech, Inc.) and FITC-conjugated goat anti-mouse IgG. For this analysis, 25  $\mu$ l of cells suspended (at  $4 \times 10^6$  cells/ml) in cell sorter buffer (PBS containing 1% FCS and 0.02%  $\text{NaN}_3$ ) were added to U-bottom microtiter wells, mixed with 100  $\mu$ l of culture supernatant or purified antibody (purified on Protein A-sepharose columns) (10  $\mu$ g /ml) in cell sorter buffer, and incubated for 30 minutes on ice. The cells were then washed and incubated with 100  $\mu$ l FITC-conjugated goat anti-mouse IgG for 30 minutes at 4°C. Cells were then washed twice, resuspended in 150  $\mu$ l of cell sorter buffer and then analyzed by FACScan (Becton Dickinson, Mountain View, CA). FACS analysis showed 8/22 supernatants were positive for anti-Apo-2 antibodies.

Figure 7 shows the FACS staining of 9D cells incubated with one of the Apo-2 antibodies, referred to as 3F11.39.7. As shown in Figure 7, the 3F11.39.7 antibody recognizes the Apo-2 receptor expressed in 9D cells.

5

#### EXAMPLE 10

##### Assay for Ability of Apo-2 Abs to Agonistically induce Apoptosis

10

Hybridoma supernatants and purified antibodies (as described in Example 9 above) were tested for activity to induce Apo-2 mediated 9D cell apoptosis. The 9D cells ( $5 \times 10^5$  cells/0.1ml) were incubated with varying concentrations of antibodies in 100  $\mu$ l complete RPMI media at 4°C for 15 minutes. The cells were then incubated for 5 minutes at 37°C and 10  $\mu$ g of goat anti-mouse IgG Fc antibody (Cappel Laboratories) in 300  $\mu$ l of complete RPMI was added to some of the cell samples. At this point, the cells were incubated overnight at 37°C and in the presence of 7% CO<sub>2</sub>. The cells were then harvested and washed once with PBS. The viability of the cells was determined by staining of FITC-annexin V binding to phosphatidylserine according to manufacturer recommendations (Clontech). The cells were washed in PBS and resuspended in 200  $\mu$ l binding buffer. Ten  $\mu$ l of annexin-V-FITC (1  $\mu$ g/ml) and 10  $\mu$ l of propidium iodide were added to the cells. After incubation for 15 minutes in the dark, the 9D cells were analyzed by FACS.

As shown in Figure 8, the 3F11.39.7 antibody (in the absence of the goat anti-mouse IgG Fc) induced apoptosis in the 9D cells as compared to the control antibodies. Agonistic activity, however, was enhanced by Apo-2 receptor cross-linking in the presence of the goat anti-mouse IgG Fc (see Figure 9). This enhanced apoptosis (Figure 9) by the combination of antibodies is comparable to the apoptotic activity of Apo-2L in 9D cells (data not shown).

35

EXAMPLE 11Assay for Antibody Ability to Block Apo-2 ligand-induced Apoptosis

5           Hybridoma supernatants and purified antibodies (as described in Example 9 above) were tested for activity to block Apo-2 ligand induced 9D cell apoptosis. The 9D cells ( $5 \times 10^5$  cells/0.1ml) were suspended in complete RPMI media (RPMI plus 10%FCS, glutamine, nonessential amino acids, penicillin, streptomycin, sodium pyruvate) and placed into individual Falcon 2052 tubes. Cells were then incubated with 10  $\mu$ g of antibodies in 200  $\mu$ l media for 15 minutes on ice. 0.2 ml of Apo-2 ligand (2.5  $\mu$ g/ml) (soluble His-tagged Apo-2L prepared as described in WO 97/25428; see also Pitti et al., supra) was suspended into complete 15 RPMI media, and then added into the tubes containing the 9D cells. The 9D cells were incubated overnight at 37°C and in the presence of 7% CO<sub>2</sub>. The incubated cells were then harvested and washed once with PBS. The viability of the cells was determined by staining of FITC-annexin V binding to phosphatidylserine according to 20 manufacturer recommendations (Clontech). Specifically, the cells were washed in PBS and resuspended in 200  $\mu$ l binding buffer. Ten  $\mu$ l of annexin-V-FITC (1  $\mu$ g/ml) and 10  $\mu$ l of propidium iodide were added to the cells. After incubation for 15 minutes in the dark, the 9D cells were analyzed by FACS.

25           The results are shown in Figure 10. Since 9D cells express more than one receptor for Apo-2L, Apo-2L can induce apoptosis in the 9D cells by interacting with either Apo-2 or the DR4 receptor. Thus, to detect any blocking activity of the Apo-2 antibodies, the interaction between DR4 and Apo-2L needed to be 30 blocked. In combination with the anti-DR4 antibody, 4H6.17.8 (ATCC HB-12455), the Apo-2 antibody 3F11.39.7 was able to block approximately 50% of apoptosis induced by Apo-2L. The remaining approximately 50% apoptotic activity is believed to be due to the agonistic activities of these two antibodies by themselves, as 35 shown in Figure 10. Accordingly, it is believed that the 3F11.39.7 antibody is a blocking Apo-2 antibody or an antibody which binds Apo-2 in a mode which competes with binding of Apo-2 ligand to Apo-2.

EXAMPLE 12ELISA Assay to Test Binding of Apo-2 Antibodies to Other  
Apo-2 Ligand Receptors

5           An ELISA was conducted to determine if the monoclonal  
antibody described in Example 9 was able to bind other known Apo-2L  
receptors beside Apo-2. Specifically, the 3F11.39.7 antibody was  
tested for binding to DR4 [Pan et al., supra], DcR1 [Sheridan et  
al., supra], and DcR2 [Marsters et al., Curr. Biol., 7:1003-1006  
10 (1997)]. The ELISA was performed essentially as described in  
Example 9 above.

The results are shown in Figure 11. The Apo-2 antibody  
3F11.39.7 bound to Apo-2. The 3F11.39.7 antibody also showed some  
cross-reactivity to DR4, but not to DcR1 or DcR2.

15

EXAMPLE 13Antibody Isotyping

The isotype of the 3F11.39.7 antibody (as described  
above) was determined by coating microtiter plates with isotype  
20 specific goat anti-mouse Ig (Fisher Biotech, Pittsburgh, PA)  
overnight at 4°C. The plates were then washed with wash buffer (as  
described in Example 9 above). The wells in the microtiter plates  
were then blocked with 200 µl of 2% bovine serum albumin (BSA) and  
incubated at room temperature for one hour. The plates were washed  
25 again three times with wash buffer. Next, 100 µl of 5 µg/ml of  
purified 3F11.39.7 antibody was added to designated wells. The  
plates were incubated at room temperature for 30 minutes and then  
50 µl HRP-conjugated goat anti-mouse IgG (as described above) was  
added to each well. The plates were incubated for 30 minutes at  
30 room temperature. The level of HRP bound to the plate was detected  
using HRP substrate as described above.

The isotyping analysis showed that the 3F11.39.7  
antibody is an IgG1 antibody.

EXAMPLE 14Single-Chain Apo-2 AntibodiesA. Antibody Phage Selection using streptavidin-coated paramagnetic beads

5           A phage library was selected using soluble biotinylated antigen and streptavidin-coated paramagnetic beads. The antigen, an Apo-2 ECD immunoadhesin prepared as described in Example 2B above, was biotinylated using IMMUNOPURE NHS-biotin (biotiny-N-hydroxy-succinimide, Pierce) according to manufacturer's  
10 instructions.

Two panning experiments were performed. The first experiment was designed to isolate phage clones specific for Apo-2 and which did not cross react with DR4 or DcR1. Three rounds of panning were carried out. For the first round, 10 µl of the  
15 Cambridge Antibody Technologies phage library were blocked with 1 ml of MPBST (3% dry milk powder, 1X PBS, 0.2% TWEEN) containing 800 µg of CD4-Ig, 300 µg DR4-Ig, and 200 µg of DcR1-Ig for 1 hour on a rotating wheel at room temperature (CD4-Ig, DR4, and DcR1 are described in Capon et al., Nature, 337:525 (1989); Pan et al.,  
20 supra; and Sheridan et al., supra). Biotinylated Apo-2 ECD immunoadhesin was then added to a final concentration of 100 nM, and phage were allowed to bind antigen for 1 hour at 37 °C. Meanwhile, 300 µl of DYNABEADS M-280, coated with streptavidin (DYNAL) were washed 3 times with 1 ml MPBST (using a DYNAL Magnetic  
25 Particle Concentrator) and then blocked for 2 hours at 37 °C with 1 ml fresh MPBST on a rotator. The beads were collected with the MPC; resuspended in 50 µl of MPBST, and added to the phage-plus-antigen solution. Mixing continued on a wheel at room temperature for 15 minutes. The DYNABEADS and attached phage were then washed  
30 a total of 7 times: 3 times with 1 ml PBS-TWEEN, once with MPBS, followed by 3 times with PBS.

Phage were eluted from the beads by incubating 5 minutes at room temperature with 300 µl of 100 mM triethylamine. The phage-containing supernatant was removed and neutralized with 150  
35 µl of 1 M Tris-HCl (pH 7.4). Neutralized phage were used to infect mid-log TG1 host cells and plated on 2YT agar supplemented with 2%

glucose and 100 µg/ml carbenicillin. After overnight growth at 30 °C, colonies were scraped into 10 ml 2YT. 50 µl of this solution was used to inoculate 25 ml of 2YT with carbenicillin and glucose and incubated, shaking, for 2 hours at 37 °C. Helper phage M13KO7 (Pharmacia) were added at a m.o.i. of 10. After adsorption, the cells were pelleted and resuspended in 25 ml of 2YT with carbenicillin (100 µg/ml) and kanamycin (50 µg/ml) and growth continued at 30 °C for 4 hours. *E. coli* were removed from the phage by centrifugation, and 1 ml of these phage (approximately  $10^{12}$  c.f.u.) were used in subsequent rounds of selection.

For the second round of selection, the 1 ml of harvested phage was adjusted to 3% dry milk, 1X PBS, 0.2% TWEEN and then 100 µg DR4-Ig, 65 µg DcR1-Ig, and 500 µg of CD4-Ig were added for blocking. For selection, biotinylated Apo-2 was added at 10 nM. Washing stringency was increased to two cycles of 7 washes.

For the third round of selection, phage were blocked with only MPBST. Biotinylated Apo-2 was added to 1 nM, and washing stringency was increased to three cycles of 7 washes. Relatively few clones were obtained in this round; therefore Pan 2B, Round 3 was performed using 5 nM of biotinylated Apo-2 with all other conditions repeated as before.

A second panning experiment was performed similarly as above except that in Rounds 1 and 2, blocking of phage solutions was conducted with MPBST containing 1.0 mg/ml CD4-Ig (no other immunoadhesins) and Round 3 was blocked with MPBST only. Biotinylated Apo-2 was added at 200 nM in Round 1, 60 nM in Round 2, and 12 nM in Round 3. At each round, phage were eluted from the magnetic beads with 300 µl of 100 nM triethylamine, then with 300 µl 0.1 M Tris-HCl (pH 7.5), and then with 300 µl glycine-0.1 M HCl (pH 2.2) containing 1 mg/ml BSA. The phage obtained from the three sequential elutions were pooled and used to infect host strain TG1 as above.

#### B. ELISA screening of selected clones

After each round of selection, individual carbenicillin-resistant colonies were screened by ELISA to identify those producing Apo-2-binding phage. Only those clones

which were positive in two or more assay formats were further studied.

Individual clones were inoculated into 2TY with 2% glucose and 100  $\mu\text{g/ml}$  carbenicillin in 96-well tissue culture plates and grown until turbid. Cultures were then infected at a m.o.i. of 10 with M12KO7 helper phage, and infected cells were transferred to 2YT media containing carbenicillin (100  $\mu\text{g/ml}$ ) and kanamycin (50  $\mu\text{g/ml}$ ) for growth overnight at 30°C with gentle shaking.

NUNC MAXISORP microtiter plates were coated with 50  $\mu\text{l}$  per well of Apo-2 ECD immunoadhesin, or CD4-IgG, at 2  $\mu\text{g/ml}$  in 50 mM carbonate buffer (pH 9.6), at 4°C overnight. After removing antigen, plates were blocked with 3% dry milk in PBS (MPBS) for 2 hours at room temperature.

Phage cultures were centrifuged and 100  $\mu\text{l}$  of phage-containing supernatants were blocked with 20  $\mu\text{l}$  of 6 x PBS / 18% dry milk for 1 hour at room temperature. Block was removed from titer plates and blocked phage added and allowed to bind for 1 hour at room temperature. After washing, phage were detected with a 1:5000 dilution of horseradish peroxidase-conjugated anti-M13 antibody (Pharmacia) in MPBS followed by 3',3',5',5'-tetramethylbenzidine (TMB). Reactions were stopped by the addition of  $\text{H}_2\text{SO}_4$  and readings taken by subtracting the  $A_{405\text{nm}}$  from the  $A_{450\text{nm}}$ .

#### 25 C. DNA fingerprinting of clones

The diversity of Apo-2-binding clones was determined by PCR amplifying the scFv insert using primers pUC19R (5'AGC GGA TAA CAA TTT CAC ACA GG 3') (SEQ. ID. NO:12) which anneals upstream of the leader sequence and fdtetseq (5'GTC GTC TTT CCA GAC GGT AGT 3') (SEQ. ID. NO:13) which anneals in the 5' end of gene III, followed by digestion with the frequent-cutting restriction enzyme BstNI.

#### DNA Fingerprinting: Protocol

35 Mix A:            dH2O                            67  $\mu\text{l}$   
                    10 x ampliTaQ buffer 10



	25 mM MgCl <sub>2</sub>	10
	DMSO, 50%	2
	forward primer	1
5	Mix B:	
	2.5 mM dNTPs	8 $\mu$ l
	AMPLITAQ	0.5
	reverse primer	1.0

90  $\mu$ l of Mix A was placed in a reaction tube and then inoculated  
 10 with a very small portion of *E. coli* colony using a yellow tip. The reaction mix was then heated in a PCR block to 98°C, for 3 minutes, removed, and placed on ice. 10  $\mu$ l Mix B was then added and the reaction mix was thermocycled at 95° C, 30 sec, 55°C 30 sec, 72°C 1 minute 20 sec, for 25 cycles in a Perkin Elmer 2400  
 15 thermocycler. 10  $\mu$ l of the resultant reaction product was then removed and run on a 1% agarose gel to test for a 1 kB band. The remaining mix was brought to 1 x BstNI reaction buffer, 5 units BstNI was added and the DNA was allowed to digest for 2 hours at 60°C. The resultant samples were then electrophoresed on a  
 20 GeneGel Excel 12.5% acrylamide gel (Pharmacia Biotech).

#### D. Sequencing of clones

The nucleotide sequence of representative clones of each fingerprint pattern were obtained. Colonies were inoculated into 50 ml of LB medium supplemented with 2% glucose and 100  $\mu$ g/ml  
 25 carbenicillin, and grown overnight at 30°C. DNA was isolated using Qiagen Tip-100s and the manufacturer's protocol and cycle sequenced with fluorescent dideoxy chain terminators (Applied Biosystems). Samples were run on an Applied Biosystems 373A Automated DNA Sequencer and sequences analyzed using the program  
 30 "Sequencher" (Gene Codes Corporation). The nucleotides sequences of selected antibodies 16E2, 20E6 and 24C4 are shown in SEQ ID NO:6, SEQ ID NO:7, and SEQ ID NO:8, respectively, (in Figures 15A, 15B and 15C respectively). The corresponding amino acid sequences of antibodies 16E2, 20E6 and 24C4 are shown in SEQ ID NO:9, SEQ ID  
 35 NO:10, and SEQ ID NO:11, respectively (and in Figure 16). In addition, Figure 16 identifies the signal region, and heavy and light chain complementarity determining regions (underlined) of these scFv molecules. The CDR regions shown in Figure 16 were

assigned according to the methods of Kabat et al., "Sequences of Proteins of Immunological Interest," NIH Publ. No. 91-3242, 5<sup>th</sup> Edition.

#### E. Purification of scFvs with (his)<sub>6</sub>

5 For protein purification of soluble antibody, *E. coli* strain 33D3 was transformed with phagemid DNA. Five ml of 2YT with carbenicillin and glucose was used to grow overnight cultures at 30°C. 2.5 ml of these cultures were diluted into 250 ml of the same media and grown to an OD<sub>600</sub> of approximately 1.2. The cells  
10 were pelleted and resuspended in 500 ml of 2YT containing IPTG (1 mM) and carbenicillin (100 µg/ml) to induce expression and grown for a further 16 hours at 22°C. Cell pellets were harvested and frozen at -20°C.

The antibodies were purified by immobilized metal  
15 chelate affinity chromatography (IMAC). Frozen pellets were resuspended in 10 ml of ice-cold shockate buffer (25 mM TRIS-HCl, 1 mM EDTA, 500 mM NaCl, 20% sucrose, 1 mM PMSF) by shaking on ice for 1 hour. Imidazole was added to 20 mM, and cell debris removed by centrifugation. The supernatants were adjusted to 1mM MgCl<sub>2</sub>  
20 and 50 mM phosphate buffer pH 7.5. Ni-NTA agarose resin from Qiagen was used according to the manufacturer's instructions. The resin was equilibrated with 50 mM sodium phosphate buffer pH 7.5, 500 mM NaCl, 20 mM imidazole, and the shockate added. Binding occurred in either a batch mode or on a gravity flow column. The  
25 resin was then washed twice with 10 bed volumes of equilibration buffer, and twice with buffer containing imidazole increased to 50mM. Elution of proteins was with 50 mM phosphate buffer pH 7.5, 500 mM NaCl and 250 mM imidazole. Excess salt and imidazole was removed on a PD-10 column (Pharmacia), and proteins were  
30 concentrated using a Centricon 10 to a volume of about 1 ml.

Concentration was estimated spectrophotometrically assuming an A280 nm of 1.0 = 0.6 mg/ml.

#### F. Assays to determine binding specificity of anti-Apo-2 scFvs

35 To evaluate the specificity of each of the scFv clones, ELISA assays were performed to evaluate binding of 16E2, 20E6 and

24C4 to Apo-2 ECD-Ig, DR4-Ig, DcR1-Ig, DcR2-Ig and CD4-Ig (described above and in Example 12).

In brief, NUNC ELISA plates were coated with 50  $\mu$ l of a 1  $\mu$ g/ml receptor-Ig immunoadhesin molecule in 0.05 M sodium carbonate buffer, pH 9.5, and allowed to incubate overnight at 4°C. Plates were then blocked with 285  $\mu$ l ELISA diluent (PBS supplemented with 0.5% BSA, 0.05% Tween 20, pH 7.4) for at least one hour at room temperature. 50  $\mu$ l of the scFvs were added to the plates in a 1:5 serial dilution and allowed to incubate for 1 hour at room temperature. After this 1 hour dilution, the plates were washed 6 times with PBS/0.05% Tween. After binding to antigen coated plates, soluble scFv was detected by adding 50  $\mu$ l of 1  $\mu$ g/ml Mab 9E10 (an anti-c-myc antibody; ATCC CRL 1729) per well and allowing the plates to incubate for 1 hour at room temperature. After washing the plates 6 times with PBS/0.05% Tween, 50  $\mu$ l of a 1:5000 dilution of horseradish peroxidase-conjugated anti-Murine IgG antibody (Cappel catalogue: 55569) in MPBS was added to the plates and allowed to incubate for 1 hour. An observable signal was generated by adding 50  $\mu$ l of 3',3',5',5'-tetramethylbenzidine (TMB) peroxidase substrate (KPL catalogue #: 50-76-00). Reactions were stopped by the addition of H<sub>2</sub>SO<sub>4</sub> and readings taken by subtracting the A<sub>405nm</sub> from the A<sub>450nm</sub>.

As illustrated in Figures 12A, 12B and 12C, the ELISA assays showed that each of these antibodies exhibited a relatively high degree of specificity for Apo-2.

Additional assays utilizing transfected cells also showed the specificity of 16E2 antibody for Apo-2. Specifically, immunohistochemistry experiments were performed to evaluate the binding specificity of the 16E2 antibody to Apo-2 and DR4-transfected CHO cells. CHO cells were transfected with vector alone or vector containing the gene for Apo-2 or DR4. The transfected cells were removed from culture plates, pelleted, and washed twice with PBS. The pellets were then resuspended in O.C.T. (Fisher), flash frozen in isopentain and LN<sub>2</sub>, and later sectioned using standard protocols. Staining of the sectioned cells was performed using a Vectastain Elite ABC kit. The sections were incubated with either anti-Apo-2 antibody 16E2 or a negative control single chain antibody. The secondary antibody

employed was either a biotinylated anti-c-myc 9E10 antibody or anti-Penta His antibody (Qiagen) followed by biotinylated anti-mouse IgG.

5 This immunohistochemistry assay showed specific staining of the Apo-2-transfected cells but not the DR4-transfected cells. The cellular staining was predominantly cytoplasmic.

#### EXAMPLE 15

##### 10 Assay for Ability of His-tagged scFvs to Agonistically induce Apoptosis

###### A. Annexin V-biotin/Streptavidin-[S-35] 96 Well Assays

Purified scFv antibodies (as described in Example 14 above) were tested for ability to induce Apo-2 mediated apoptosis.

15 In brief, SK-MES-1 cells (human lung carcinoma cell line; ATCC HTB 58) or HCT 116 cells (human colon carcinoma cell line; ATCC CCL 247) ( $4 \times 10^4$  cells/well) were aliquoted into 96 well plates in assay medium (1:1 mixture of phenol-red free Dulbecco modified Eagle medium and phenol-red free Ham's F-12 nutrient  
20 mixture supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 ug/ml streptomycin) and allowed to attach overnight at 37°C. The media was then removed and 0.1 ml of assay medium containing scFv at a final concentration of 50 ug/ml (16E2 or 20E6) was added to the wells (serial dilutions of 1:2  
25 performed in the plates) and allowed to incubate for 1 hour at room temperature. Other single chain antibodies were used as negative controls: an anti-tissue factor scFv clone, 7D5, or a scFv referred to as 19B8. After the 1 hour incubation with scFv antibody, 0.1 ml of 10 ug/ml anti-His (Qiagen, cat. No. 1007671) or anti-c-myc  
30 antibodies were added to the appropriate wells. Wells not receiving a crosslinking antibody received media alone. The plates were then allowed to incubate for 30 minutes at room temperature. After the 30 minutes incubation, 0.1 ml of 10 ug/ml goat anti-mouse IgG (ICN cst. No. 67-029) was added to the appropriate wells.  
35 Wells not receiving anti-IgG antibody received media alone. The plates were then placed in an incubator for 15 minutes to allow the pH to return to 7.0. For positive controls, a 2 ug/ml solution of Apo-2 ligand (Apo-2L) (prepared as described in Example 11) in potassium phosphate buffer at pH 7.0 was added to the appropriate

wells, with serial 2 fold dilutions carried out in the plate. The negative control wells received media alone. The cells were then incubated overnight at 37°C in the presence of 5% CO<sub>2</sub>. 0.05 ml of annexin V-biotin (1 ug/ml) in 2X Ca<sup>2+</sup> binding buffer (NeXins B.V.) was then added to the wells and then allowed to mix on a shaker for 30 minutes. 0.05 ml of streptavidin-[S-35] (final concentration of 2.5 x 10<sup>4</sup> cpm/well) (Amersham) in 2X Ca<sup>2+</sup> binding buffer was then added to the wells and then allowed to mix on a shaker for 30 minutes. The plates were then sealed and centrifuged for 4 minutes at 1500 rpm. To assess the extent of apoptosis, the plates were then counted on a Trialux Microbeta Counter (Wallace) to obtain cpm values corresponding to Annexin-V binding.

As shown in Figures 13C and 14B, the 16E2 and 20E6 antibodies agonistically induced apoptosis in SK-MES-1 cells.

#### B. Crystal Violet Assays

In addition to the annexin V-biotin/streptavidin-[S-35] assay described above, scFv antibodies (as described in Example 14 above) were tested for activity to induce Apo-2 mediated apoptosis via assays utilizing crystal violet.

In brief, the SK-MES-1 cells were plated at 4x10<sup>4</sup> cells/well in assay medium (described in Section A above) and allowed to attach overnight at 37°C. The medium was removed and 0.1ml of assay medium containing scFv (as described in Section A above) at a final concentration of 50 µg/ml was added to the appropriate wells (wells without scFv added receive a media change). Selected wells received "pre-complexed" samples in which 10 ug/ml scFv 16E2 was combined with 100 ug/ml anti-His antibody for 5 hours at 4° C with continuous mixing before addition to the plate. The plates were allowed to incubate for 1 hour at room temperature.

The scFv medium was removed and 0.1 ml of 10 µg/ml anti-His (Qiagen, cat. no. 1007671) or anti-c-myc antibodies diluted in assay medium was added to the wells (wells without crosslinker receive a media change.) The plates were then allowed to incubate for 30 minutes at room temperature.

The medium was then removed and 0.1 ml of 10 µg/ml Goat anti-Mouse IgG (Fc Fragment specific-ICN cst. no. 67-029) diluted

in assay medium was added to the appropriate wells (wells without anti-Fc receive a media change). The plates were then placed in the incubator for 15 minutes to allow the pH to return to 7.0.

Apo-2L (stock at 100 µg/ml in potassium phosphate buffer pH 7.0) was diluted to 2 µg/ml and 0.1ml was added to the appropriate wells. Serial two-fold dilutions were carried down the plate. The plates were then incubated overnight at 37°C.

All medium was removed from the wells and the plates were then flooded with crystal violet solution. The plates were allowed to stain for 15 minutes. The crystal violet was removed by flooding the plates with running tap water. The plates were then allowed to dry overnight.

The plates were read on an SLT plate reader at 540nm and the data analyzed using an Excel macro and 4p-fit.

As shown in Figures 13A, 13B, 14A and 14B, the 16E2 and 20E6 antibodies agonistically induced apoptosis in SK-MES-1 cells.

#### EXAMPLE 16

##### Assay for Ability of gD-tagged scFvs to Agonistically Induce Apoptosis

A purified gD-tagged form of 16E2 scFv was tested for ability to induce Apo-2 mediated apoptosis in a crystal violet assay as described in Example 15 above.

##### A. Construction of scFv with gD tag

The Sfi I to Not I fragment of the scFv form of 16E2 was subcloned into a derivative of pAK19 (Carter et al., Methods: A Companion to Methods in Enzymology, 3:183-192 (1991)) containing the *phoA* promoter and stII signal sequence rather than the lacZ promoter and hybrid signal sequence of the original library. For ease of purification, a DNA fragment coding for 12 amino acids (met-ala-asp-pro-asn-arg-phe-arg-gly-lys-asp-leu SEQ ID NO:14) derived from herpes simplex virus type 1 glycoprotein D (Lasky et al., DNA, 3:23-29 (1984)) was synthesized and inserted at the 3' end of the VL domain in place of the (his)<sub>6</sub> and *c-myc* epitope originally present in the Cambridge Antibody Technologies library clones.

##### B. Expression in E. coli

The plasmid containing the gene for scFv 16E2-gD was transformed into *E. coli* strain 33D3 for expression in shake flask cultures. 5 ml of 2YT with carbenicillin and glucose was used to grow overnight cultures at 30° C. 2.5 ml of these cultures were diluted into 250 ml of the same medium and grown to an OD<sub>600</sub> of approximately 1.0. The cells were pelleted and resuspended in 500 ml of Modified AP-5 Minimal Media containing carbenicillin (100 µg/ml) and grown for an additional 16 hours at 30° C. The cells were then pelleted and frozen.

#### C. Purification of scFv with gD tag

Frozen cell paste was resuspended at 1gm/10ml of shockate buffer (25 mM Tris-HCl, 1 mM EDTA, 500 mM NaCl, 20% sucrose, 1 mM PMSF, pH 7.2) and gently agitated 4 hours on ice. The cell suspension was then processed through a Polytron microfluidizer (Brinkman). Cell debris was removed by centrifugation at 10,000 x g for 30 minutes. After filtration through a 0.22 micron filter, the supernatant was loaded onto an affinity column (2.5 x 9.0 cm) consisting of an anti-gD antibody 5B6 (Paborsky et al., Protein Engineering, 3:547-553 (1990)) coupled to CNBr Sepharose which had been equilibrated with PBS. The column was washed 18 hours with PBS until the absorbance of the column effluent was equivalent to baseline. All steps were done at 4° C at a linear flow rate of 25 cm/hour. Elution was performed with 0.1 M acetic acid, 0.5 M NaCl, pH 2.9. Column fractions were monitored by absorbance at 280 nm and peak fractions pooled, neutralized with 1.0 M Tris, pH 8.0, dialyzed against PBS and sterile filtered. The resultant protein preparations were analyzed by non-reducing SDS-PAGE.

#### D. Crystal Violet Assay

The apoptosis assay was performed essentially as described in Example 15(B) above except that samples were serially diluted 1:3 in the plates and the 16E2-gD tagged antibody was tested in addition to two other preparations of 16E2 scFv (referred to as Prep. A and Prep. B in Figure 14C). The results of the assay showing apoptosis induction in SK-MES-1 cells by 16E2-gD antibody are illustrated in Figure 14C.

\* \* \* \* \*

Deposit of Material

The following materials have been deposited with the American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia, USA (ATCC):

<u>Material</u>	<u>ATCC Dep. No.</u>	<u>Deposit Date</u>
pRK5-Apo-2	209021	May 8, 1997
3F11.39.7	HB-12456	January 13, 1998

10

This deposit was made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of a viable culture of the deposit for 30 years from the date of deposit. The deposit will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Genentech, Inc. and ATCC, which assures permanent and unrestricted availability of the progeny of the culture of the deposit to the public upon issuance of the pertinent U.S. patent or upon laying open to the public of any U.S. or foreign patent application, whichever comes first, and assures availability of the progeny to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC Section 122 and the Commissioner's rules pursuant thereto (including 37 CFR Section 1.14 with particular reference to 886 OG 638).

The assignee of the present application has agreed that if a culture of the materials on deposit should die or be lost or destroyed when cultivated under suitable conditions, the materials will be promptly replaced on notification with another of the same. Availability of the deposited material is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by the construct deposited, since the deposited embodiment is intended



as a single illustration of certain aspects of the invention and any constructs that are functionally equivalent are within the scope of this invention. The deposit of material herein does not constitute an admission that the written description herein  
5 contained is inadequate to enable the practice of any aspect of the invention, including the best mode thereof, nor is it to be construed as limiting the scope of the claims to the specific illustrations that it represents. Indeed, various modifications of the invention in addition to those shown and described herein will  
10 become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

5 (i) APPLICANT: Adams, Camilia W.  
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Kim, Kyung J.

10 (ii) TITLE OF INVENTION: Apo-2 Receptor

(iii) NUMBER OF SEQUENCES: 14

15 (iv) CORRESPONDENCE ADDRESS:  
(A) ADDRESSEE: Genentech, Inc.  
(B) STREET: 1 DNA Way  
(C) CITY: South San Francisco  
(D) STATE: California  
(E) COUNTRY: USA  
20 (F) ZIP: 94080

(v) COMPUTER READABLE FORM:  
(A) MEDIUM TYPE: 3.5 inch, 1.44 Mb floppy disk  
(B) COMPUTER: IBM PC compatible  
25 (C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: WinPatin (Genentech)

(vi) CURRENT APPLICATION DATA:  
(A) APPLICATION NUMBER:  
30 (B) FILING DATE:  
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:  
(A) NAME: Marschang, Diane L.  
35 (B) REGISTRATION NUMBER: 35,600  
(C) REFERENCE/DOCKET NUMBER: P1101R2

(ix) TELECOMMUNICATION INFORMATION:  
(A) TELEPHONE: 650/225-5416  
40 (B) TELEFAX: 650/952-9881

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 411 amino acids  
45 (B) TYPE: Amino Acid  
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

50 Met Glu Gln Arg Gly Gln Asn Ala Pro Ala Ala Ser Gly Ala Arg  
1 5 10 15

Lys Arg His Gly Pro Gly Pro Arg Glu Ala Arg Gly Ala Arg Pro  
20 25 30

55 Gly Leu Arg Val Pro Lys Thr Leu Val Leu Val Val Ala Ala Val  
35 40 45

60 Leu Leu Leu Val Ser Ala Glu Ser Ala Leu Ile Thr Gln Gln Asp  
50 55 60

Leu Ala Pro Gln Gln Arg Ala Ala Pro Gln Gln Lys Arg Ser Ser  
65 70 75

	Pro Ser Glu Gly Leu Cys Pro Pro Gly His His Ile Ser Glu Asp	80	85	90
5	Gly Arg Asp Cys Ile Ser Cys Lys Tyr Gly Gln Asp Tyr Ser Thr	95	100	105
	His Trp Asn Asp Leu Leu Phe Cys Leu Arg Cys Thr Arg Cys Asp	110	115	120
10	Ser Gly Glu Val Glu Leu Ser Pro Cys Thr Thr Thr Arg Asn Thr	125	130	135
	Val Cys Gln Cys Glu Glu Gly Thr Phe Arg Glu Glu Asp Ser Pro	140	145	150
15	Glu Met Cys Arg Lys Cys Arg Thr Gly Cys Pro Arg Gly Met Val	155	160	165
20	Lys Val Gly Asp Cys Thr Pro Trp Ser Asp Ile Glu Cys Val His	170	175	180
	Lys Glu Ser Gly Ile Ile Ile Gly Val Thr Val Ala Ala Val Val	185	190	195
25	Leu Ile Val Ala Val Phe Val Cys Lys Ser Leu Leu Trp Lys Lys	200	205	210
	Val Leu Pro Tyr Leu Lys Gly Ile Cys Ser Gly Gly Gly Gly Asp	215	220	225
30	Pro Glu Arg Val Asp Arg Ser Ser Gln Arg Pro Gly Ala Glu Asp	230	235	240
35	Asn Val Leu Asn Glu Ile Val Ser Ile Leu Gln Pro Thr Gln Val	245	250	255
	Pro Glu Gln Glu Met Glu Val Gln Glu Pro Ala Glu Pro Thr Gly	260	265	270
40	Val Asn Met Leu Ser Pro Gly Glu Ser Glu His Leu Leu Glu Pro	275	280	285
	Ala Glu Ala Glu Arg Ser Gln Arg Arg Arg Leu Leu Val Pro Ala	290	295	300
45	Asn Glu Gly Asp Pro Thr Glu Thr Leu Arg Gln Cys Phe Asp Asp	305	310	315
50	Phe Ala Asp Leu Val Pro Phe Asp Ser Trp Glu Pro Leu Met Arg	320	325	330
	Lys Leu Gly Leu Met Asp Asn Glu Ile Lys Val Ala Lys Ala Glu	335	340	345
55	Ala Ala Gly His Arg Asp Thr Leu Tyr Thr Met Leu Ile Lys Trp	350	355	360
	Val Asn Lys Thr Gly Arg Asp Ala Ser Val His Thr Leu Leu Asp	365	370	375
60	Ala Leu Glu Thr Leu Gly Glu Arg Leu Ala Lys Gln Lys Ile Glu	380	385	390
	Asp His Leu Leu Ser Ser Gly Lys Phe Met Tyr Leu Glu Gly Asn			

Ala Asp Ser Ala Xaa Ser  
410 411

5

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

10

- (A) LENGTH: 1799 base pairs  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

15

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CCCACGCGTC CGCATAAATC AGCACGCGGC CGGAGAACCC CGCAATCTCT 50  
GCGCCACAA AATACACCGA CGATGCCCGA TCTACTTTAA GGGCTGAAAC 100  
CCACGGGCCT GAGAGACTAT AAGAGCGTTC CCTACCGCC ATG GAA 145  
Met Glu  
1  
CAA CGG GGA CAG AAC GCC CCG GCC GCT TCG GGG GCC CGG 184  
Gln Arg Gly Gln Asn Ala Pro Ala Ala Ser Gly Ala Arg  
5 10 15  
AAA AGG CAC GGC CCA GGA CCC AGG GAG GCG CGG GGA GCC 223  
Lys Arg His Gly Pro Gly Pro Arg Glu Ala Arg Gly Ala  
20 25  
AGG CCT GGG CTC CGG GTC CCC AAG ACC CTT GTG CTC GTT 262  
Arg Pro Gly Leu Arg Val Pro Lys Thr Leu Val Leu Val  
30 35 40  
GTC GCC GCG GTC CTG CTG TTG GTC TCA GCT GAG TCT GCT 301  
Val Ala Ala Val Leu Leu Leu Val Ser Ala Glu Ser Ala  
45 50  
CTG ATC ACC CAA CAA GAC CTA GCT CCC CAG CAG AGA GCG 340  
Leu Ile Thr Gln Gln Asp Leu Ala Pro Gln Gln Arg Ala  
55 60 65  
GCC CCA CAA CAA AAG AGG TCC AGC CCC TCA GAG GGA TTG 379  
Ala Pro Gln Gln Lys Arg Ser Ser Pro Ser Glu Gly Leu  
70 75 80  
TGT CCA CCT GGA CAC CAT ATC TCA GAA GAC GGT AGA GAT 418  
Cys Pro Pro Gly His His Ile Ser Glu Asp Gly Arg Asp  
85 90  
TGC ATC TCC TGC AAA TAT GGA CAG GAC TAT AGC ACT CAC 457  
Cys Ile Ser Cys Lys Tyr Gly Gln Asp Tyr Ser Thr His  
95 100 105  
TGG AAT GAC CTC CTT TTC TGC TTG CGC TGC ACC AGG TGT 496  
Trp Asn Asp Leu Leu Phe Cys Leu Arg Cys Thr Arg Cys  
110 115  
GAT TCA GGT GAA GTG GAG CTA AGT CCC TGC ACC ACG ACC 535  
Asp Ser Gly Glu Val Glu Leu Ser Pro Cys Thr Thr Thr  
120 125 130

60

AGA AAC ACA GTG TGT CAG TGC GAA GAA GGC ACC TTC CGG 574  
 Arg Asn Thr Val Cys Gln Cys Glu Glu Gly Thr Phe Arg  
 135 140 145

5 GAA GAA GAT TCT CCT GAG ATG TGC CGG AAG TGC CGC ACA 613  
 Glu Glu Asp Ser Pro Glu Met Cys Arg Lys Cys Arg Thr  
 150 155

10 GGG TGT CCC AGA GGG ATG GTC AAG GTC GGT GAT TGT ACA 652  
 Gly Cys Pro Arg Gly Met Val Lys Val Gly Asp Cys Thr  
 160 165 170

15 CCC TGG AGT GAC ATC GAA TGT GTC CAC AAA GAA TCA GGC 691  
 Pro Trp Ser Asp Ile Glu Cys Val His Lys Glu Ser Gly  
 175 180

20 ATC ATC ATA GGA GTC ACA GTT GCA GCC GTA GTC TTG ATT 730  
 Ile Ile Ile Gly Val Thr Val Ala Ala Val Val Leu Ile  
 185 190 195

GTG GCT GTG TTT GTT TGC AAG TCT TTA CTG TGG AAG AAA 769  
 Val Ala Val Phe Val Cys Lys Ser Leu Leu Trp Lys Lys  
 200 205 210

25 GTC CTT CCT TAC CTG AAA GGC ATC TGC TCA GGT GGT GGT 808  
 Val Leu Pro Tyr Leu Lys Gly Ile Cys Ser Gly Gly Gly  
 215 220

30 GGG GAC CCT GAG CGT GTG GAC AGA AGC TCA CAA CGA CCT 847  
 Gly Asp Pro Glu Arg Val Asp Arg Ser Ser Gln Arg Pro  
 225 230 235

35 GGG GCT GAG GAC AAT GTC CTC AAT GAG ATC GTG AGT ATC 886  
 Gly Ala Glu Asp Asn Val Leu Asn Glu Ile Val Ser Ile  
 240 245

40 TTG CAG CCC ACC CAG GTC CCT GAG CAG GAA ATG GAA GTC 925  
 Leu Gln Pro Thr Gln Val Pro Glu Gln Glu Met Glu Val  
 250 255 260

CAG GAG CCA GCA GAG CCA ACA GGT GTC AAC ATG TTG TCC 964  
 Gln Glu Pro Ala Glu Pro Thr Gly Val Asn Met Leu Ser  
 265 270 275

45 CCC GGG GAG TCA GAG CAT CTG CTG GAA CCG GCA GAA GCT 1003  
 Pro Gly Glu Ser Glu His Leu Leu Glu Pro Ala Glu Ala  
 280 285

50 GAA AGG TCT CAG AGG AGG AGG CTG CTG GTT CCA GCA AAT 1042  
 Glu Arg Ser Gln Arg Arg Arg Leu Leu Val Pro Ala Asn  
 290 295 300

55 GAA GGT GAT CCC ACT GAG ACT CTG AGA CAG TGC TTC GAT 1081  
 Glu Gly Asp Pro Thr Glu Thr Leu Arg Gln Cys Phe Asp  
 305 310

60 GAC TTT GCA GAC TTG GTG CCC TTT GAC TCC TGG GAG CCG 1120  
 Asp Phe Ala Asp Leu Val Pro Phe Asp Ser Trp Glu Pro  
 315 320 325

CTC ATG AGG AAG TTG GGC CTC ATG GAC AAT GAG ATA AAG 1159  
 Leu Met Arg Lys Leu Gly Leu Met Asp Asn Glu Ile Lys  
 330 335 340

GTG GCT AAA GCT GAG GCA GCG GGC CAC AGG GAC ACC TTG 1198  
 Val Ala Lys Ala Glu Ala Ala Gly His Arg Asp Thr Leu  
 345 350

5 TAC ACG ATG CTG ATA AAG TGG GTC AAC AAA ACC GGG CGA 1237  
 Tyr Thr Met Leu Ile Lys Trp Val Asn Lys Thr Gly Arg  
 355 360 365

10 GAT GCC TCT GTC CAC ACC CTG CTG GAT GCC TTG GAG ACG 1276  
 Asp Ala Ser Val His Thr Leu Leu Asp Ala Leu Glu Thr  
 370 375

15 CTG GGA GAG AGA CTT GCC AAG CAG AAG ATT GAG GAC CAC 1315  
 Leu Gly Glu Arg Leu Ala Lys Gln Lys Ile Glu Asp His  
 380 385 390

20 TTG TTG AGC TCT GGA AAG TTC ATG TAT CTA GAA GGT AAT 1354  
 Leu Leu Ser Ser Gly Lys Phe Met Tyr Leu Glu Gly Asn  
 395 400 405

GCA GAC TCT GCC WTG TCC TAAGTGTG ATTCTCTTCA GGAAGTGAGA 1400  
 Ala Asp Ser Ala Xaa Ser  
 410 411

25 CCTTCCCTGG TTTACCTTTT TTCTGGAAAA AGCCCAACTG GACTCCAGTC 1450

AGTAGGAAAG TGCCACAATT GTCACATGAC CGGTACTGGA AGAAACTCTC 1500

30 CCATCCAACA TCACCCAGTG GATGGAACAT CCTGTAACTT TTCACTGCAC 1550

TTGGCATTAT TTTTATAAGC TGAATGTGAT AATAAGGACA CTATGGAAAT 1600

GTCTGGATCA TTCCGTTTGT GCGTACTTTG AGATTTGGTT TGGGATGTCA 1650

35 TTGTTTTTAC AGCACTTTTT TATCCTAATG TAAATGCTTT ATTTATTTAT 1700

TTGGGCTACA TTGTAAGATC CATCTACAAA AAAAAAAAAA AAAAAAAAAAG 1750

40 GGCGGCCGCG ACTCTAGAGT CGACCTGCAG AAGCTTGGCC GCCATGGCC 1799

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

45 (A) LENGTH: 70 base pairs  
 (B) TYPE: Nucleic Acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

50

GGGAGCCGCT CATGAGGAAG TTGGGCCTCA TGGACAATGA GATAAAGGTG 50

GCTAAAGCTG AGGCAGCGGG 70

55

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

60 (A) LENGTH: 29 base pairs  
 (B) TYPE: Nucleic Acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ATCAGGGACT TTCCGCTGGG GACTTTCCG 29

5 (2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 30 base pairs  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

15

AGGATGGGAA GTGTGTGATA TATCCTTGAT 30

(2) INFORMATION FOR SEQ ID NO:6:

20 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 930 base pairs  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

30 ATG ACC ATG ATT ACG CCA AGC TTT GGA GCC TTT TTT 36  
Met Thr Met Ile Thr Pro Ser Phe Gly Ala Phe Phe  
1 5 10

35 TTG GAG ATT TTC AAC GTG AAA AAA TTA TTA TTC GCA ATT 75  
Leu Glu Ile Phe Asn Val Lys Lys Leu Leu Phe Ala Ile  
15 20 25

CCT TTA GTT GTT CCT TTC TAT GCG GCC CAG CCG GCC ATG 114  
Pro Leu Val Val Pro Phe Tyr Ala Ala Gln Pro Ala Met  
30 35

40 GCC GAG GTG CAG CTG GTG CAG TCT GGG GGA GGT GTG GAA 153  
Ala Glu Val Gln Leu Val Gln Ser Gly Gly Gly Val Glu  
40 45 50

45 CGG CCG GGG GGG TCC CTG AGA CTC TCC TGT GCA GCC TCT 192  
Arg Pro Gly Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser  
55 60

50 GGA TTC ACC TTT GAT GAT TAT GGC ATG AGC TGG GTC CGC 231  
Gly Phe Thr Phe Asp Asp Tyr Gly Met Ser Trp Val Arg  
65 70 75

55 CAA GCT CCA GGG AAG GGG CTG GAG TGG GTC TCT GGT ATT 270  
Gln Ala Pro Gly Lys Gly Leu Glu Trp Val Ser Gly Ile  
80 85 90

AAT TGG AAT GGT GGT AGC ACA GGA TAT GCA GAC TCT GTG 309  
Asn Trp Asn Gly Gly Ser Thr Gly Tyr Ala Asp Ser Val  
95 100

60 AAG GGC CGA GTC ACC ATC TCC AGA GAC AAC GCC AAG AAC 348  
Lys Gly Arg Val Thr Ile Ser Arg Asp Asn Ala Lys Asn  
105 110 115

TCC CTG TAT CTG CAA ATG AAC AGC CTG AGA GCC GAG GAC 387  
 Ser Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp  
 120 125

5 ACG GCC GTA TAT TAC TGT GCG AAA ATC CTG GGT GCC GGA 426  
 Thr Ala Val Tyr Tyr Cys Ala Lys Ile Leu Gly Ala Gly  
 130 135 140

10 CGG GGC TGG TAC TTC GAT CTC TGG GGG AAG GGG ACC ACG 465  
 Arg Gly Trp Tyr Phe Asp Leu Trp Gly Lys Gly Thr Thr  
 145 150 155

15 GTC ACC GTC TCG AGT GGT GGA GGC GGT TCA GGC GGA GGT 504  
 Val Thr Val Ser Ser Gly Gly Gly Ser Gly Gly Gly  
 160 165

20 GGC AGC GGC GGT GGC GGA TCG TCT GAG CTG ACT CAG GAC 543  
 Gly Ser Gly Gly Gly Gly Ser Ser Glu Leu Thr Gln Asp  
 170 175 180

CCT GCT GTG TCT GTG GCC TTG GGA CAG ACA GTC AGG ATC 582  
 Pro Ala Val Ser Val Ala Leu Gly Gln Thr Val Arg Ile  
 185 190

25 ACA TGC CAA GGA GAC AGC CTC AGA AGC TAT TAT GCA AGC 621  
 Thr Cys Gln Gly Asp Ser Leu Arg Ser Tyr Tyr Ala Ser  
 195 200 205

30 TGG TAC CAG CAG AAG CCA GGA CAG GCC CCT GTA CTT GTC 660  
 Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Val Leu Val  
 210 215 220

35 ATC TAT GGT AAA AAC AAC CGG CCC TCA GGG ATC CCA GAC 699  
 Ile Tyr Gly Lys Asn Asn Arg Pro Ser Gly Ile Pro Asp  
 225 230

40 CGA TTC TCT GGC TCC AGC TCA GGA AAC ACA GCT TCC TTG 738  
 Arg Phe Ser Gly Ser Ser Ser Gly Asn Thr Ala Ser Leu  
 235 240 245

ACC ATC ACT GGG GCT CAG GCG GAA GAT GAG GCT GAC TAT 777  
 Thr Ile Thr Gly Ala Gln Ala Glu Asp Glu Ala Asp Tyr  
 250 255

45 TAC TGT AAC TCC CGG GAC AGC AGT GGT AAC CAT GTG GTA 816  
 Tyr Cys Asn Ser Arg Asp Ser Ser Gly Asn His Val Val  
 260 265 270

50 TTC GGC GGA GGG ACC AAG CTG ACC GTC CTA GGT GCG GCC 855  
 Phe Gly Gly Gly Thr Lys Leu Thr Val Leu Gly Ala Ala  
 275 280 285

55 GCA CAT CAT CAT CAC CAT CAC GGG GCC GCA GAA CAA AAA 894  
 Ala His His His His His His Gly Ala Ala Glu Gln Lys  
 290 295

60 CTC ATC TCA GAA GAG GAT CTG AAT GGG GCC GCA TAG 930  
 Leu Ile Ser Glu Glu Asp Leu Asn Gly Ala Ala  
 300 305 309

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 939 base pairs



(B) TYPE: Nucleic Acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGY: Linear

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

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      ATG ACC ATG ATT ACG CCA AGC TTT GGA GCC TTT TTT 36
      Met Thr Met Ile Thr Pro Ser Phe Gly Ala Phe Phe
10      1                      5                      10

      TTG GAG ATT TTC AAC GTG AAA AAA TTA TTA TTC GCA ATT 75
      Leu Glu Ile Phe Asn Val Lys Lys Leu Leu Phe Ala Ile
      15                      20                      25

15      CCT TTA GTT GTT CCT TTC TAT GCG GCC CAG CCG GCC ATG 114
      Pro Leu Val Val Pro Phe Tyr Ala Ala Gln Pro Ala Met
      30                      35

20      GCC GGG GTG CAG CTG GTG GAG TCT GGG GGA GGC TTG GTC 153
      Ala Gly Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val
      40                      45                      50

      CAG CCT GGG GGG TCC CTG AGA CTC TCC TGT GCA GCC TCT 192
25      Gln Pro Gly Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser
      55                      60

      GGA TTC ACC TTT AGT AGC TAT TGG ATG AGC TGG GTC CGC 231
30      Gly Phe Thr Phe Ser Ser Tyr Trp Met Ser Trp Val Arg
      65                      70                      75

      CAG GCT CCA GGG AAG GGG CTG GAG TGG GTG GCC AAC ATA 270
      Gln Ala Pro Gly Lys Gly Leu Glu Trp Val Ala Asn Ile
      80                      85                      90

35      AAG CAA GAT GGA AGT GAG AAA TAC TAT GTG GAC TCT GTG 309
      Lys Gln Asp Gly Ser Glu Lys Tyr Tyr Val Asp Ser Val
      95                      100

40      AAG GGC CGA TTC ACC ATC TCC AGA GAC AAC GCC AAG AAC 348
      Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn
      105                      110                      115

      TCA CTG TAT CTG CAA ATG AAC AGC CTG AGA GCC GAG GAC 387
45      Ser Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
      120                      125

      ACG GCT GTG TAT TAC TGT GCG AGA GAT CTT TTA AAG GTC 426
50      Thr Ala Val Tyr Tyr Cys Ala Arg Asp Leu Leu Lys Val
      130                      135                      140

      AAG GGC AGC TCG TCT GGG TGG TTC GAC CCC TGG GGG AGA 465
      Lys Gly Ser Ser Ser Gly Trp Phe Asp Pro Trp Gly Arg
      145                      150                      155

55      GGG ACC ACG GTC ACC GTC TCG AGT GGT GGA GGC GGT TCA 504
      Gly Thr Thr Val Thr Val Ser Ser Gly Gly Gly Gly Ser
      160                      165

60      GGC GGA GGT GGT AGC GGC GGT GGC GGA TCG TCT GAG CTG 543
      Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Ser Glu Leu
      170                      175                      180

      ACT CAG GAC CCT GCT GTG TCT GTG GCC TTG GGA CAG ACA 582

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Thr Gln Asp Pro Ala Val Ser Val Ala Leu Gly Gln Thr  
185 190

5 GTC AGG ATC ACA TGC CAA GGA GAC AGC CTC AGA AGC TAT 621  
Val Arg Ile Thr Cys Gln Gly Asp Ser Leu Arg Ser Tyr  
195 200 205

10 TAT GCA AGC TGG TAC CAG CAG AAG CCA GGA CAG GCC CCT 660  
Tyr Ala Ser Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro  
210 215 220

GTA CTT GTC ATC TAT GGT AAA AAC AAC CGG CCC TCA GGG 699  
Val Leu Val Ile Tyr Gly Lys Asn Asn Arg Pro Ser Gly  
225 230

15 ATC CCA GAC CGA TTC TCT GGC TCC AGC TCA GGA AAC ACA 738  
Ile Pro Asp Arg Phe Ser Gly Ser Ser Ser Gly Asn Thr  
235 240 245

20 GCT TCC TTG ACC ATC ACT GGG GCT CAG GCG GAA GAT GAG 777  
Ala Ser Leu Thr Ile Thr Gly Ala Gln Ala Glu Asp Glu  
250 255

25 GCT GAC TAT TAC TGT AAC TCC CGG GAC AGC AGT GGT AAC 816  
Ala Asp Tyr Tyr Cys Asn Ser Arg Asp Ser Ser Gly Asn  
260 265 270

30 CAT GTG GTA TTC GGC GGA GGG ACC AAG CTG ACC GTC CTA 855  
His Val Val Phe Gly Gly Gly Thr Lys Leu Thr Val Leu  
275 280 285

GGT GCG GCC GCA CAT CAT CAT CAC CAT CAC GGG GCC GCA 894  
Gly Ala Ala Ala His His His His His His Gly Ala Ala  
290 295

35 GAA CAA AAA CTC ATC TCA GAA GAG GAT CTG AAT GGG GCC 933  
Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu Asn Gly Ala  
300 305 310

40 GCA TAG 939  
Ala  
312

## (2) INFORMATION FOR SEQ ID NO:8:

- 45 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 933 base pairs  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
50 (D) TOPOLOGY: Linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

55 ATG ACC ATG ATT ACG CCA AGC TTT GGA GCC TTT TTT 36  
Met Thr Met Ile Thr Pro Ser Phe Gly Ala Phe Phe  
1 5 10

60 TTG GAG ATT TTC AAC GTG AAA AAA TTA TTA TTC GCA ATT 75  
Leu Glu Ile Phe Asn Val Lys Lys Leu Leu Phe Ala Ile  
15 20 25

CCT TTA GTT GTT CCT TTC TAT GCG GCC CAG CCG GCC ATG 114  
Pro Leu Val Val Pro Phe Tyr Ala Ala Gln Pro Ala Met

5 GCC CAG GTG CAG CTG GTG CAG TCT GGG GGA GGC GTG GTC 153  
 Ala Gln Val Gln Leu Val Gln Ser Gly Gly Gly Val Val  
 40 45 50

10 CAG CCT GGG CGG TCC CTG AGA CTC TCC TGT GCA GCT TCT 192  
 Gln Pro Gly Arg Ser Leu Arg Leu Ser Cys Ala Ala Ser  
 55 60

15 GGG TTC ATT TTC AGT AGT TAT GGG ATG CAC TGG GTC CGC 231  
 Gly Phe Ile Phe Ser Ser Tyr Gly Met His Trp Val Arg  
 65 70 75

20 CAG GCT CCA GGC AAG GGG CTG GAG TGG GTG GCA GGT ATT 270  
 Gln Ala Pro Gly Lys Gly Leu Glu Trp Val Ala Gly Ile  
 80 85 90

25 TTT TAT GAT GGA GGT AAT AAA TAC TAT GCA GAC TCC GTG 309  
 Phe Tyr Asp Gly Gly Asn Lys Tyr Tyr Ala Asp Ser Val  
 95 100

30 AAG GGC CGA TTC ACC ATC TCC AGA GAC AAT TCC AAG AAC 348  
 Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn  
 105 110 115

35 ACG CTG TAT CTG CAA ATG AAC AGC CTG AGA GCT GAG GAC 387  
 Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp  
 120 125

40 ACG GCT GTG TAT TAC TGT GCG AGA GAT AGG GGC TAC TAC 426  
 Thr Ala Val Tyr Tyr Cys Ala Arg Asp Arg Gly Tyr Tyr  
 130 135 140

45 TAC ATG GAC GTC TGG GGC AAA GGG ACC ACG GTC ACC GTC 465  
 Tyr Met Asp Val Trp Gly Lys Gly Thr Thr Val Thr Val  
 145 150 155

50 TCC TCA GGT GGA GGC GGT TCA GGC GGA GGT GGC TCT GGC 504  
 Ser Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly  
 160 165

55 GGT GGC GGA TCG CAG TCT GTG TTG ACG CAG CCG CCC TCA 543  
 Gly Gly Gly Ser Gln Ser Val Leu Thr Gln Pro Pro Ser  
 170 175 180

60 GTG TCT GGG GCC CCA GGA CAG AGG GTC ACC ATC TCC TGC 582  
 Val Ser Gly Ala Pro Gly Gln Arg Val Thr Ile Ser Cys  
 185 190

65 ACT GGG AGA AGC TCC AAC ATC GGG GCA GGT CAT GAT GTA 621  
 Thr Gly Arg Ser Ser Asn Ile Gly Ala Gly His Asp Val  
 195 200 205

70 CAC TGG TAC CAG CAA CTT CCA GGA ACA GCC CCC AAA CTC 660  
 His Trp Tyr Gln Gln Leu Pro Gly Thr Ala Pro Lys Leu  
 210 215 220

75 CTC ATC TAT GAT GAC AGC AAT CGG CCC TCA GGG GTC CCT 699  
 Leu Ile Tyr Asp Asp Ser Asn Arg Pro Ser Gly Val Pro  
 225 230

80 GAC CGA TTC TCT GGC TCC AGG TCT GGC ACC TCA GCC TCC 738  
 Asp Arg Phe Ser Gly Ser Arg Ser Gly Thr Ser Ala Ser

235

240

245

CTG GCC ATC ACT GGG CTC CAG GCT GAA GAT GAG GCT GAT 777  
 Leu Ala Ile Thr Gly Leu Gln Ala Glu Asp Glu Ala Asp  
 250 255

TAT TAC TGC CAG TCC TAT GAC AGC AGC CTG AGG GGT TCG 816  
 Tyr Tyr Cys Gln Ser Tyr Asp Ser Ser Leu Arg Gly Ser  
 260 265 270

GTA TTC GGC GGA GGG ACC AAG GTC ACT GTC CTA GGT GCG 855  
 Val Phe Gly Gly Gly Thr Lys Val Thr Val Leu Gly Ala  
 275 280 285

GCC GCA CAT CAT CAT CAC CAT CAC GGG GCC GCA GAA CAA 894  
 Ala Ala His His His His His His Gly Ala Ala Glu Gln  
 290 295

AAA CTC ATC TCA GAA GAG GAT CTG AAT GGG GCC GCA 930  
 Lys Leu Ile Ser Glu Glu Asp Leu Asn Gly Ala Ala  
 300 305 310

TAG 933

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 309 amino acids  
 (B) TYPE: Amino Acid  
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Met Thr Met Ile Thr Pro Ser Phe Gly Ala Phe Phe Leu Glu Ile  
 1 5 10 15

Phe Asn Val Lys Lys Leu Leu Phe Ala Ile Pro Leu Val Val Pro  
 20 25 30

Phe Tyr Ala Ala Gln Pro Ala Met Ala Glu Val Gln Leu Val Gln  
 35 40 45

Ser Gly Gly Gly Val Glu Arg Pro Gly Gly Ser Leu Arg Leu Ser  
 50 55 60

Cys Ala Ala Ser Gly Phe Thr Phe Asp Asp Tyr Gly Met Ser Trp  
 65 70 75

Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val Ser Gly Ile  
 80 85 90

Asn Trp Asn Gly Gly Ser Thr Gly Tyr Ala Asp Ser Val Lys Gly  
 95 100 105

Arg Val Thr Ile Ser Arg Asp Asn Ala Lys Asn Ser Leu Tyr Leu  
 110 115 120

Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys  
 125 130 135

Ala Lys Ile Leu Gly Ala Gly Arg Gly Trp Tyr Phe Asp Leu Trp  
 140 145 150

Gly Lys Gly Thr Thr Val Thr Val Ser Ser Gly Gly Gly Ser

	155	160	165
	Gly Gly Gly Gly Ser Gly Gly Gly Gly	Ser Ser Glu Leu Thr Gln	
	170	175	180
5	Asp Pro Ala Val Ser Val Ala Leu Gly	Gln Thr Val Arg Ile Thr	
	185	190	195
10	Cys Gln Gly Asp Ser Leu Arg Ser Tyr	Tyr Ala Ser Trp Tyr Gln	
	200	205	210
	Gln Lys Pro Gly Gln Ala Pro Val Leu	Val Ile Tyr Gly Lys Asn	
	215	220	225
15	Asn Arg Pro Ser Gly Ile Pro Asp Arg	Phe Ser Gly Ser Ser Ser	
	230	235	240
	Gly Asn Thr Ala Ser Leu Thr Ile Thr	Gly Ala Gln Ala Glu Asp	
20	245	250	255
	Glu Ala Asp Tyr Tyr Cys Asn Ser Arg	Asp Ser Ser Gly Asn His	
	260	265	270
25	Val Val Phe Gly Gly Gly Thr Lys Leu	Thr Val Leu Gly Ala Ala	
	275	280	285
	Ala His His His His His His Gly Ala	Ala Glu Gln Lys Leu Ile	
	290	295	300
30	Ser Glu Glu Asp Leu Asn Gly Ala Ala		
	305	309	

## (2) INFORMATION FOR SEQ ID NO:10:

35 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 312 amino acids  
 (B) TYPE: Amino Acid  
 (D) TOPOLOGY: Linear

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

	Met Thr Met Ile Thr Pro Ser Phe Gly Ala Phe Phe Leu Glu Ile	
	1 5 10 15	
45	Phe Asn Val Lys Lys Leu Leu Phe Ala Ile Pro Leu Val Val Pro	
	20 25 30	
	Phe Tyr Ala Ala Gln Pro Ala Met Ala Gly Val Gln Leu Val Glu	
50	35 40 45	
	Ser Gly Gly Gly Leu Val Gln Pro Gly Gly Ser Leu Arg Leu Ser	
	50 55 60	
55	Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser Tyr Trp Met Ser Trp	
	65 70 75	
	Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val Ala Asn Ile	
	80 85 90	
60	Lys Gln Asp Gly Ser Glu Lys Tyr Tyr Val Asp Ser Val Lys Gly	
	95 100 105	
	Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Ser Leu Tyr Leu	
	110 115 120	

	Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys	
	125	130 135
5	Ala Arg Asp Leu Leu Lys Val Lys Gly Ser Ser Ser Gly Trp Phe	
	140	145 150
	Asp Pro Trp Gly Arg Gly Thr Thr Val Thr Val Ser Ser Gly Gly	
	155	160 165
10	Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Ser Glu	
	170	175 180
	Leu Thr Gln Asp Pro Ala Val Ser Val Ala Leu Gly Gln Thr Val	
15	185	190 195
	Arg Ile Thr Cys Gln Gly Asp Ser Leu Arg Ser Tyr Tyr Ala Ser	
	200	205 210
20	Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Val Leu Val Ile Tyr	
	215	220 225
	Gly Lys Asn Asn Arg Pro Ser Gly Ile Pro Asp Arg Phe Ser Gly	
	230	235 240
25	Ser Ser Ser Gly Asn Thr Ala Ser Leu Thr Ile Thr Gly Ala Gln	
	245	250 255
	Ala Glu Asp Glu Ala Asp Tyr Tyr Cys Asn Ser Arg Asp Ser Ser	
30	260	265 270
	Gly Asn His Val Val Phe Gly Gly Gly Thr Lys Leu Thr Val Leu	
	275	280 285
35	Gly Ala Ala Ala His His His His His His Gly Ala Ala Glu Gln	
	290	295 300
	Lys Leu Ile Ser Glu Glu Asp Leu Asn Gly Ala Ala	
	305	310 312

## (2) INFORMATION FOR SEQ ID NO:11:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 310 amino acids  
 (B) TYPE: Amino Acid  
 (D) TOPOLOGY: Linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

50	Met Thr Met Ile Thr Pro Ser Phe Gly Ala Phe Phe Leu Glu Ile	
	1	5 10 15
	Phe Asn Val Lys Lys Leu Leu Phe Ala Ile Pro Leu Val Val Pro	
	20	25 30
55	Phe Tyr Ala Ala Gln Pro Ala Met Ala Gln Val Gln Leu Val Gln	
	35	40 45
	Ser Gly Gly Gly Val Val Gln Pro Gly Arg Ser Leu Arg Leu Ser	
60	50	55 60
	Cys Ala Ala Ser Gly Phe Ile Phe Ser Ser Tyr Gly Met His Trp	
	65	70 75

	Val	Arg	Gln	Ala	Pro	Gly	Lys	Gly	Leu	Glu	Trp	Val	Ala	Gly	Ile	
						80				85					90	
5	Phe	Tyr	Asp	Gly	Gly	Asn	Lys	Tyr	Tyr	Ala	Asp	Ser	Val	Lys	Gly	
						95				100					105	
	Arg	Phe	Thr	Ile	Ser	Arg	Asp	Asn	Ser	Lys	Asn	Thr	Leu	Tyr	Leu	
						110				115					120	
10	Gln	Met	Asn	Ser	Leu	Arg	Ala	Glu	Asp	Thr	Ala	Val	Tyr	Tyr	Cys	
						125				130					135	
	Ala	Arg	Asp	Arg	Gly	Tyr	Tyr	Tyr	Met	Asp	Val	Trp	Gly	Lys	Gly	
15						140				145					150	
	Thr	Thr	Val	Thr	Val	Ser	Ser	Gly	Gly	Gly	Gly	Ser	Gly	Gly	Gly	
						155				160					165	
20	Gly	Ser	Gly	Gly	Gly	Gly	Ser	Gln	Ser	Val	Leu	Thr	Gln	Pro	Pro	
						170				175					180	
	Ser	Val	Ser	Gly	Ala	Pro	Gly	Gln	Arg	Val	Thr	Ile	Ser	Cys	Thr	
						185				190					195	
25	Gly	Arg	Ser	Ser	Asn	Ile	Gly	Ala	Gly	His	Asp	Val	His	Trp	Tyr	
						200				205					210	
	Gln	Gln	Leu	Pro	Gly	Thr	Ala	Pro	Lys	Leu	Leu	Ile	Tyr	Asp	Asp	
30						215				220					225	
	Ser	Asn	Arg	Pro	Ser	Gly	Val	Pro	Asp	Arg	Phe	Ser	Gly	Ser	Arg	
						230				235					240	
35	Ser	Gly	Thr	Ser	Ala	Ser	Leu	Ala	Ile	Thr	Gly	Leu	Gln	Ala	Glu	
						245				250					255	
	Asp	Glu	Ala	Asp	Tyr	Tyr	Cys	Gln	Ser	Tyr	Asp	Ser	Ser	Leu	Arg	
						260				265					270	
40	Gly	Ser	Val	Phe	Gly	Gly	Gly	Thr	Lys	Val	Thr	Val	Leu	Gly	Ala	
						275				280					285	
	Ala	Ala	His	His	His	His	His	His	Gly	Ala	Ala	Glu	Gln	Lys	Leu	
45						290				295					300	
	Ile	Ser	Glu	Glu	Asp	Leu	Asn	Gly	Ala	Ala						
						305				310						

## (2) INFORMATION FOR SEQ ID NO:12:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 base pairs

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

60 AGCGGATAAC AATTCACAC AGG 23

## (2) INFORMATION FOR SEQ ID NO:13:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GTCGTCTTTC CAGACGGTAG T 21

10

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 amino acids  
(B) TYPE: Amino Acid  
(D) TOPOLOGY: Linear

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

20 Met Ala Asp Pro Asn Arg Phe Arg Gly Lys Asp Leu  
1 5 10 12



## What is claimed is:

1. Isolated Apo-2 polypeptide having at least 80% amino acid sequence identity with native sequence Apo-2 polypeptide comprising amino acid residues 1 to 411 of SEQ ID NO:1.
- 5 2. The Apo-2 polypeptide of claim 1 wherein said polypeptide has at least 90% amino acid sequence identity.
3. The Apo-2 polypeptide of claim 2 wherein said polypeptide has at least 95% amino acid sequence identity.
4. Isolated Apo-2 polypeptide comprising amino acid residues 1 to  
10 411 of SEQ ID NO:1.
5. Isolated extracellular domain sequence of Apo-2 polypeptide comprising amino acid residues 54 to 182 of SEQ ID NO:1.
6. The extracellular domain sequence of claim 5 comprising amino acid residues 1 to 182 of SEQ ID NO:1.
- 15 7. Isolated death domain sequence of Apo-2 polypeptide comprising amino acid residues 324 to 391 of SEQ ID NO:1.
8. A chimeric molecule comprising the Apo-2 polypeptide of claim 1 or the extracellular domain sequence of claim 5 fused to a heterologous amino acid sequence.
- 20 9. The chimeric molecule of claim 8 wherein said heterologous amino acid sequence is an epitope tag sequence.
10. The chimeric molecule of claim 8 wherein said heterologous amino acid sequence is an immunoglobulin sequence.
11. The chimeric molecule of claim 10 wherein said immunoglobulin  
25 sequence is an IgG.
12. Isolated nucleic acid comprising a DNA encoding the polypeptide of claim 1, the extracellular domain sequence of claim 5, or the death domain sequence of claim 7.
13. The nucleic acid of claim 12 wherein said DNA encodes an Apo-2  
30 polypeptide comprising amino acid residues 1 to 411 of SEQ ID NO:1.
14. A vector comprising the nucleic acid of claim 12.
15. The vector of claim 14 operably linked to control sequences recognized by a host cell transformed with the vector.
- 35 16. The vector of claim 14 comprising ATCC deposit accession number 209021.
17. A host cell comprising the vector of claim 14.
18. The host cell of claim 17 comprising a CHO cell.
19. The host cell of claim 17 comprising *E. coli*.

20. The host cell of claim 17 comprising a yeast cell.
21. A process of producing an Apo-2 polypeptide comprising culturing the host cell of claim 17 under conditions sufficient to express Apo-2 polypeptide and recovering the expressed Apo-2 polypeptide from the culture.
22. An Apo-2 polypeptide which is obtained or obtainable by expressing the polypeptide encoded by the cDNA insert in ATCC deposit accession number 209021.
23. A non-human, transgenic animal which contains cells that express DNA encoding Apo-2 polypeptide.
24. The animal of claim 23 which is a mouse or rat.
25. A non-human, knockout animal which contains cells having an altered gene encoding Apo-2 polypeptide.
26. The animal of claim 25 which is a mouse or rat.
27. An antibody which specifically binds to an Apo-2 polypeptide.
28. The antibody of claim 27 which is a monoclonal antibody.
29. The antibody of claim 27 comprising an agonistic antibody.
30. The antibody of claim 27 comprising a blocking antibody.
31. The antibody of claim 24 comprising a chimeric antibody.
32. The antibody of claim 28 wherein said antibody is an IgG antibody.
33. The antibody of claim 28 wherein said antibody comprises an Fab fragment.
34. The antibody of claim 28 wherein said antibody comprises a scFv fragment.
35. The antibody of claim 28 wherein said antibody comprises a F(ab')<sub>2</sub> fragment.
36. The antibody of claim 27 wherein said antibody comprises a human antibody.
37. The antibody of claim 28 having the biological characteristics of the monoclonal antibody produced by the hybridoma cell line deposited as ATCC accession number HB-12456.
38. The antibody of claim 28 wherein the antibody binds to the same epitope as the epitope to which the monoclonal antibody produced by the hybridoma cell line deposited as ATCC accession number HB-12456 binds.
39. A hybridoma cell line which produces the antibody of claim 28.
40. The hybridoma cell line deposited as ATCC accession number

HB-12456.

41. The monoclonal antibody produced by the hybridoma cell line deposited as ATCC accession number HB-12456.
42. The antibody of claim 27 wherein said antibody is a single-chain antibody.
43. The antibody of claim 42 wherein said antibody comprises the 16E2 antibody.
44. The antibody of claim 42 wherein said antibody comprises the 20E6 antibody.
45. The antibody of claim 42 wherein said antibody comprises the 24C4 antibody.
46. The antibody of claim 42 wherein said antibody is fused to an epitope tag sequence.
47. A chimeric molecule comprising the antibody of claim 27 fused to a heterologous amino acid sequence.
48. The chimeric molecule of claim 47 wherein said heterologous amino acid sequence comprises an immunoglobulin sequence.
49. A dimeric molecule comprising the Apo-2 antibody of claim 27 and a heterologous antibody.
50. A homodimeric molecule comprising a first Apo-2 antibody and a second Apo-2 antibody.
51. Isolated nucleic acid comprising DNA encoding the Apo-2 antibody of claim 43.
52. Isolated nucleic acid comprising DNA encoding the antibody of claim 44.
53. Isolated nucleic acid comprising DNA encoding the antibody of claim 45.
54. A vector comprising the nucleic acid of claim 51, 52, or 53.
55. A host cell comprising the vector of claim 54.
56. A method of producing an Apo-2 antibody comprising culturing the host cell of claim 55 under conditions wherein the DNA is expressed.
57. A composition comprising the antibody of claim 27 and a carrier.
58. The composition of claim 57 wherein said carrier is a pharmaceutically-acceptable carrier.
59. A method of inducing apoptosis in mammalian cancer cells comprising exposing mammalian cancer cells to an effective amount of the Apo-2 agonistic antibody of claim 29.

60. The method of claim 59 wherein said agonistic antibody comprises a single-chain antibody.
61. A method of treating mammalian cancer cells comprising exposing mammalian cancer cells to an agent which activates Apo-2.
- 5 62. The method of claim 61 wherein said agent comprises an agonistic Apo-2 antibody.
63. An article of manufacture comprising a container and a composition contained within said container, wherein the composition includes Apo-2 polypeptide or Apo-2 antibody.
- 10 64. The article of manufacture of claim 63 further comprising instructions for using the Apo-2 polypeptide or Apo-2 antibody *in vivo* or *ex vivo*.

Abstract of the Disclosure

Novel polypeptides, designated Apo-2, which are capable of modulating apoptosis are provided. Compositions including Apo-2 chimeras, nucleic acid encoding Apo-2, and antibodies to Apo-2 are  
5 also provided.

# Fig. 1

1 CCCACGGCTC GCATAAATC AGCAGCGGC CGGAGAACCC CGCAATCTCT GCGCCACAA AATACACCGA CGATGCCCGA TCTACTTTAA GGGCTGAAC  
 GGGTCCGCAG GCGTATTAG TCGTGGCCG GCTCTTGG GCGTTAGAGA CCGGGTGT TATGTGGCT GCTACGGGCT AGATGAAATT CCCGACTTTC  
 101 CCACGGGCCT GAGAGACTAT AAGAGCGTTC CCTACCGCCA TGAACAACG GGGACAGAAC GCGCCGGCGG CTTCGGGGGG CCGGAAAAGG CACGGCCAG  
 GGTGCCCGGA CTCTCTGATA TTCTCGCAAG GGATGGCGGT ACCTTGTTGC CCCTGTCTTG CCGGGCCCGG GAAGCCCCCG GGCCTTTTCC GTGCCGGGTC  
 1 GACCCAGGGA GCGCGGGGA GCGAGCCCTG GCGTCCGGT CCCAAGACC CTGTGTCTCG TTGTGCGCGG GGTCTCTCTG TTGGTCTCAG CTGAGTCTGC  
 CTGGGTCCCT CCGGCCCT CCGTCCGAC CCGAGGCCCA GGGTCTCTG GAACAGGAGC AACAGCGCGG CCAGGACGAC AACAGAGTC GACTCAGACG  
 22 ProArg1 uAlaArgGly AlaArgProG lyLeuArgVa lProLysThr LeuValLeuV alValAlaAl aValLeuLeu LeuValSera laGlusEraLa  
 301 TCTGATCACC CAACAAGACC TAGCTCCCA GCAGAGAGCG GCGCCACAAC AAAGAGGTC CAGCCCCCTCA GAGGATTGT GTCCACCTGG ACACCATATC  
 AGACTAGTGG GTTGTCTGG ATCGAGGGGT CGTCTCTCG CCGGTCTTG TTTCTCCAG GTCCGGGAGT CTCCTAACA CAGGTGGACC TGTGGTATAG  
 55 LeuileThr GlnGlnAspL euAlaProG1 nGlnArgAla AlaProGlnG lNlysArgSe rSerProSer GluGlyLeuc ysProProG1 yHisHisile  
 401 TCAGAAGACG GTAGAGATTG CATCTCCTGC AATATGGAC AGGACTATAG CACTCACTGG AATGACCTCC TTTTCTGCTT GCGTGCACC AGGTGTGATT  
 AGTCTTCTGC CATCTCTAAC GTAGAGGACG TTTATACCTG TCCTGATATC GTGAGTGACC TTACTGGAGG AAAAGACGAA CCGGACGTGG TCCACACTAA  
 88 SerGluaspG lyArgAspCy sileSerCys LysTyrGlyG lNAspTyrSe rThrHisTrp AsnAspLeuL eupheCysLe uArgCysThr ArgCysAspSer  
 501 CAGGTGAAGT GGAGCTAAGT CCCTGCACCA CGACAGAAA CACAGTGTGT CAGTGCGAAG AAGGCACCTT CCGGAAAGAA GATTCTCCTG AGATGTGCCG  
 GTCCACTTCA CCTCGATTCA GGGACGTGGT CCGTCTCTT GTGTACACA GTACCGCTTC TTCCGTGAA GCGCTTCTT CTAAGAGGAC TCTACACGGC  
 122 GlyGluVa lGluLeuser ProCysThrt hrThrArgAs nThrValCys GlnCysGluG luGlyThrPh eArgGluGlu AspSerProG luMetCysArg  
 601 GAAGTCCCGC ACAGGGTGTG CCAGAGGGAT GGTCAAGGTC GGTGATTGTA CACCTGGAG TCACATCGAA TGTGTCCACA AAGAATCAGG CATCATCAT  
 CTTACAGGCG GTGCCACAG GGTCTCCCTA CCAGTTCCAG CCACCTAACAT GTGGACCTC ACTGTAGCTT ACACAGGTGT TTCTTAGTCC GTAGTAGTAT  
 155 LysCysArg ThrGlyCysP roArgGlyMe tValLysVal GlyAspCysT hrProTirpse rAspIleGlu CysValHisL ysGluSerG lYileIleile  
 701 GGAGTCACAG TTGCAGCCGT AGTCTTGATT GTGGCTGTGT TTGTTTGCA GTCTTTACTG TGAAGAAAG TCCTTCTCTTA CCTGAAAGGC ATCTGCTCAG  
 CCTCAGTGTG AACGTGGCA TCAGAACTAA CACCGACACA AACAAAGTT CAGAAATGAC ACCTTCTTTC AGGAAGGAAT GGACTTTCCG TAGACGAGTC  
 188 GlyValThrV alAlaAlaVa lValLeuile ValAlaValP heValCysLy sSerLeuLeu TriPlysLysv alLeuProTy rLeuLysGly ileCysSerGly  
 801 GTGGTGGTGG GGACCTGAG CGTGTGGACA GAAGCTCACA ACGACCTGGG GCTGAGGACA ATGTCTCTCA TGAGATCGTG AGTATCTTGC AGCCACCCCA  
 CACCAACACC CCTGGGACTC GCACACCTGT CTTGAGTGT GTCTGGACCC CGACTCTGT TACAGGAGTT ACTCTAGCAC TCATAGAACG TCGGGTGGGT  
 222 GlyGlyG lYaspProGlu ArgValAspA rgSerSerG lNArgProGly AlaGluAspA snValLeuAs nGluileVal SerileLeug lnProThrGln  
 901 GTTCCCTGAG CAGGAAATGG AAGTCCAGGA GCCAGCAGAG CCAACAGGTG TCAACATGTT GTCCCGCGG GAGTCAGAGC ATCTGTCTGA ACCGGCAGAA  
 CCAGGGACTC GTCTTTACC TTCAGGTCTT CCGTCTCTC GGTGTCCAC AGTTGTACAA CAGGGGGGCC CTCAGTCTCG TAGACGACCT TGGCCGTCTT  
 255 ValProGlu GlnGluMetG luValGlnG lUpProAlaGlu ProThrGlyV alasnMetle uSerProGly GluSerGluH lsLeuLeuG lUpProAlaGlu  
 1001 GCTGAAAGGT CTCAGAGGAG GAGGCTGCTG GTTCCAGCAA ATGAAGGTGA TGCCACTGAG ACTCTGAGAC AGTGCTTGA TGACTTTGCA GACTTGTGTC  
 CGACTTTCCA GAGTCTCTC CTCGACGAC CAAGGTCTGTT TACTTCCACT AGGTGACTC TGAGACTCTG TCACGAAGCT ACTGAAACGT CTGAACCCAG  
 288 AlaGluArgS erGlnArgAr gArgLeuLeu ValProAlaA snGluGlyAs pProThrGlu ThrLeuArgg lncysPheAs pAspPheAla AspLeuValPro

1101 CCTTGACTC CTGGGAGCCG CTCATGAGGA AGTTGGGCCT CATGGACAAT GAGATAAAGG TGGCTAAAGC TGAGGCAGCG GCCACAGGG ACACCTTGTA  
GAAACTGAG GACCTCGGC GAGTACTCCT TCAACCCGGA GTACCTGTTA CTCTATTTC ACCGATTTCG ACTCCGTCCG CCGGTGTCCC TGTTGAACAT  
322 PheAsps<sup>o</sup> FTpGlupro LeuMetArgL ysLeuglyLe uMetAspAsn Gluilelysv alalalybAl aGluAlaAla GlyHisArga sPThrLeutyF  
1201 CACGATGCTG ATAAAGTGGG TCAACAAAAC CGGGCGAGAT GCCTCTGTCC ACACCTGTCT GGATGCCTTG GAGACGCTGG GAGAGAGACT TGCCAAGCAG  
GAGCTACGAC TATTTCACCC AGTTGTTTTG GCCCGCTCTA CGGAGACAGG TGTGGGACGA CCTACGGAAC CTCTCGGACC CTCTCTCTGA ACGGTCTGTC  
355 ThrMetLeu IleLysTrpV alasnLysTh rGlyArgasp AlaservAlH lstrLeuLe uAspAlaLeu GluThrLeug lyGluArgLe uAlaLysGln  
1301 AAGATTGAGG ACCACTTGTG GAGCTCTGGA AAGTTCTATGT ATCTAGAAGG TAATGCAGAC TCTGCCWTGT CCTAAGTGTG ATTCTCTTCA GGAAGTGAGA  
TTCTAACTCC TGGTGAACAA CTCGAGACCT TTCAAGTACA TAGATCTTCC ATTACGTCTG AGACGGAACA GGATTTCACAC TAAGAGAAGT CCTTCACTCT  
388 LysileGluA sPHisLeuLe uSerSerGly LysPheMetT yrLeuGluGl yAsnAlaasp SerAlaXqqS erOG\*  
1401 CCTTCCCTGG TTTACCTTTT TTCTGGAAAA AGCCCAACTG GACTCCAGTC AGTAGGAAAG TGCCACAATT GTCACATGAC CGGTACTGGA AGAAACTCTC  
GGAAGGGACC AAATGGAAAA AAGACCTTTT TCGGGTTGAC CTGAGGTCAG TCATCCTTTC ACGGTGTTAA CAGTGTACTG GCCATGACCT TCTTTGAGAG  
1501 CCATCCAACA TCACCCAGTG GATGGAACAT CCTGTAACTT TTCACTGCAC TTGGCATTAT TTTTATAAGC TGAATGTGAT AATAAGGACA CTATGGAAAT  
GGTAGGTTGT AGTGGGTCAC CTACCTTGTA GGACATTGAA AAGTGACGTG AACCGTAATA AAAATATTTCG ACTTACACTA TTATTCTCTGT GATACCTTTA  
1601 GTCTGGATCA TTCCGTTTGT GCGTACTTTG AGATTGTTGT TGGGATGTCA TTGTTTTTCAC AGCACTTTT TATCCTAATG TAAATGCTTT ATTTATTTAT  
CAGACCTAGT AAGGCAACA CGCATGAAAC TCTAAACCAA ACCCTACAGT AACAAAAGTG TCGTGAAAAA ATAGGATTAC ATTTACGAAA TAAATAAATA  
1701 TTGGGGTACA TTGTAAGATC CATCTACAAA AAAAAAAAAA AAAAAAAAAA GCGGCGCGCG ACTCTAGAGT CGACCTGCAG AAGCTTGGCC GCCATGGCC  
AACCCTAGT AACATTCTAG GTAGATGTTT TTTTTTTTTT TTTTTTTTTT CCGCGCGCGC TGAGATCTCA GCTGGACGTC TTCGAACCGG CGGTACCGG

Fig. 1 (cont.)

Fig. 2 A

1 MEORGONAPAAAGARKRHGPGPREARGARGLRVPKTLVLVAVLLLVSAESALITQQD  
 61 LAPQORAAAPQOKRSSPSEGLCPPGHISEDGRDCISCKYQDYSTHWNDDLFLCLRCRCD  
 121 SGEVELSPCTTTRNTVCQCEGTFREEDSPCMCRKCRGCPRGMVKVGDCIPWSDIECVH  
 181 KESGIIIGVTVAAVLIVAVEVCKSLIMKKVLPYLGICSGGGGDPERVDRSSQRPGEAD  
 241 NVLNEIVSILQPTQVPEQEMEVEQPAEPTGVNMLSPGESEHLLLEPAEARSQRRRLVPA  
 301 NEGDPTEITLROCFDDFADLVFPDSWEPLMRKLGMDNEIKVAKAEAAAGHRDITLYTMLIKW  
 361 VNKTGRDASVHTLLDALETIGERLAKQKIEDHLLSSGKFMYLEGNADSALS

Fig. 2 B

Apo2	E A D L V P P E D S W E P L M R K L G L M D N E E K V A K A E A A - - G H R D T T
DR4	E R N I V P E D S W D Q L M R Q E D D T K N E I D V V R A G T A - - G P G D A Y
Apo3/DR3	V M A A V P A R R N K E E F V R T L G L R E A E I E A V E V E I G R - - F H D Q Q
TNFR1	V V E N V P P L R N K E E F V R R L G L S D H E I D R L E L Q N G R - C L R E A Q
Fas/Apo1	I A G V M T L S Q V K G E V E R K N G V N E A K L D E I K N D N V Q D T A E Q K V
Apo2	T M L I K W V A N K T G R D - A S V H T D L D A L E E T L G E B L A K Q K I E D
DR4	V A M L M K W V N K T G R N - A S I H T D L D A L E R M E E R H A K E K I Q D
Apo3/DR3	V E M L K R R R Q Q P - A - A G L G A V Y A A I E R N G L D D G C V E D L R S
TNFR1	V S M L A T N R R R T P P R E A T L E L G R V I R D M D L L G C L E D T E E
Fas/Apo1	- Q L L R N W H Q L H G K K E A Y - D T I I K D E K K A N L C T L A E K I Q T



Fig. 3

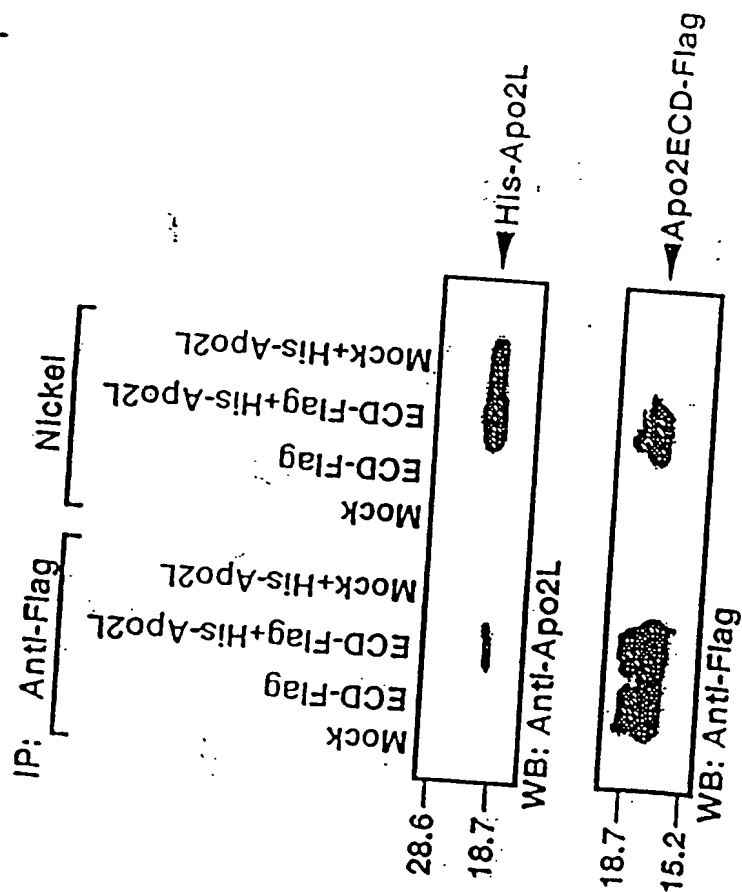
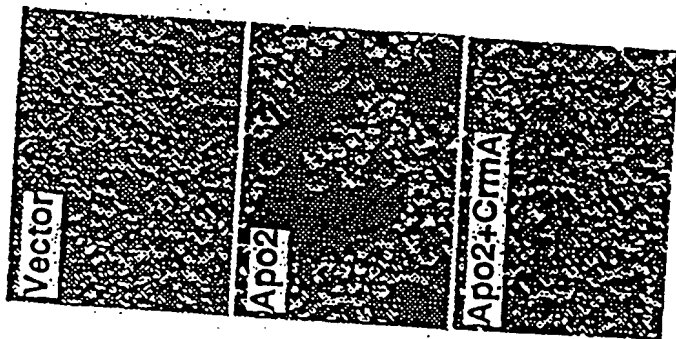
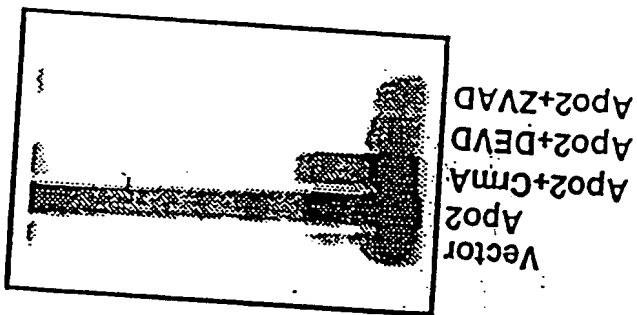


Fig. 4

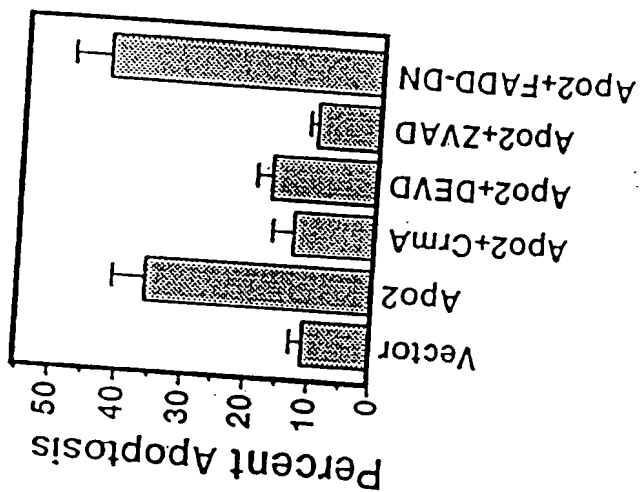
4A



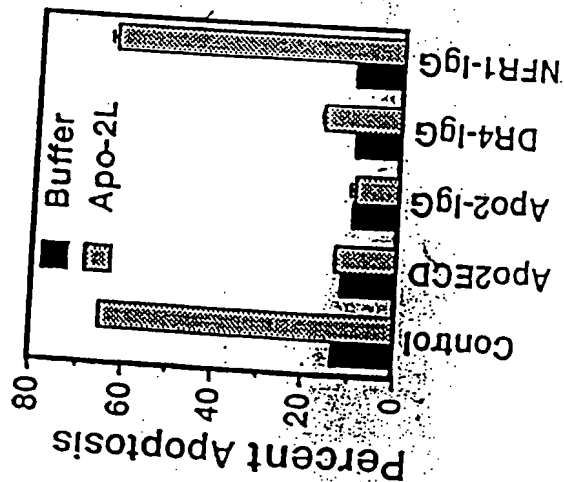
4B



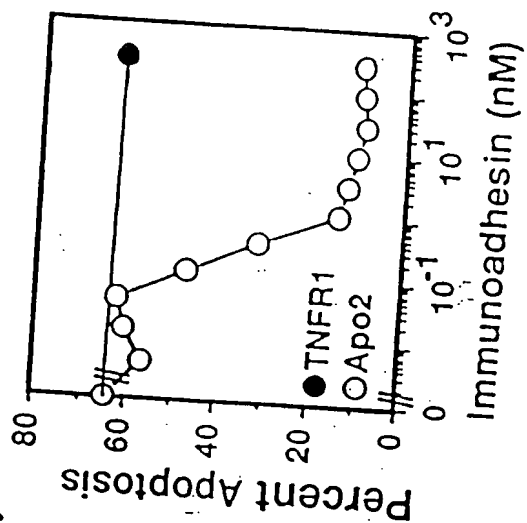
4C



4D



4E



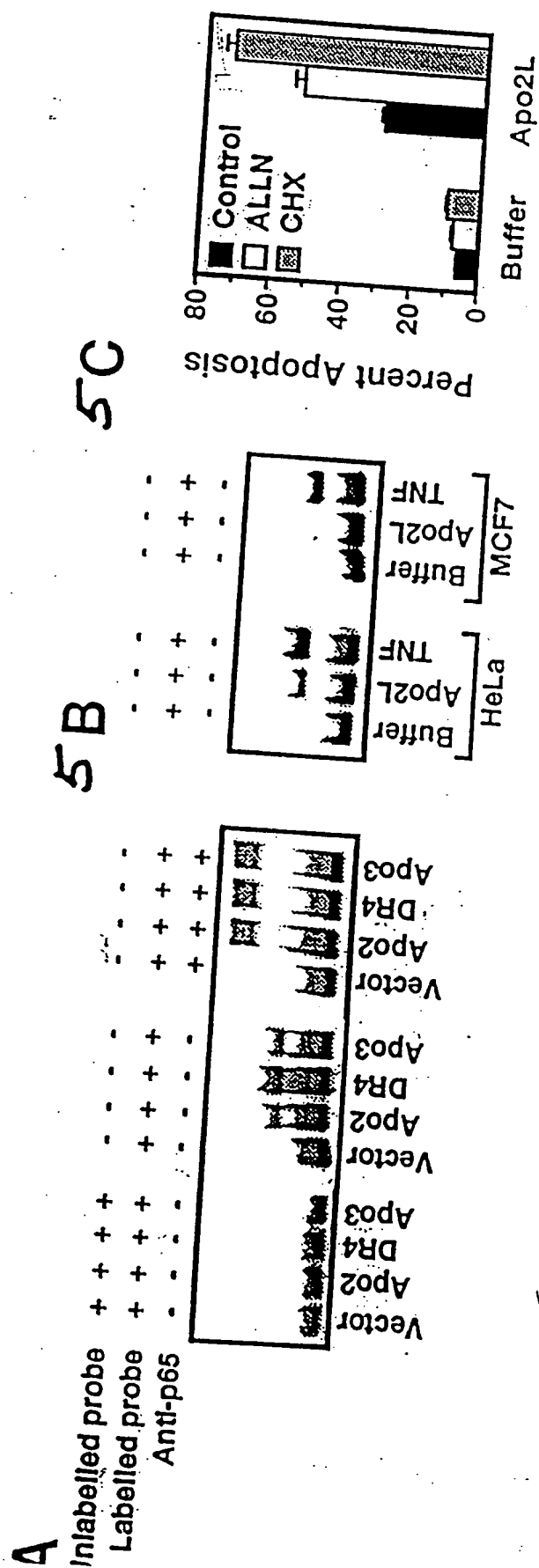
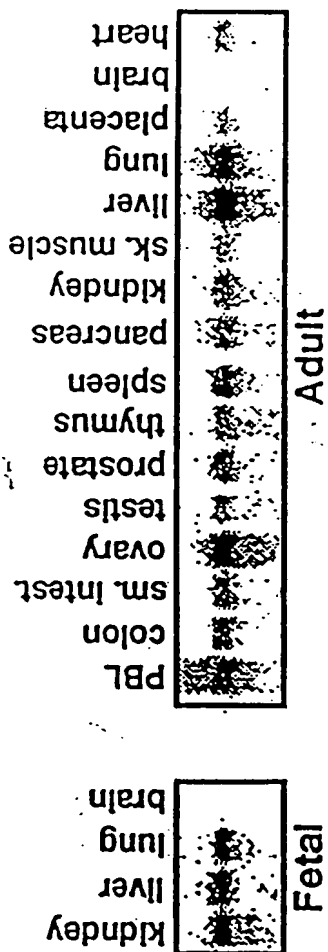


Fig. 5

FIG. 6A



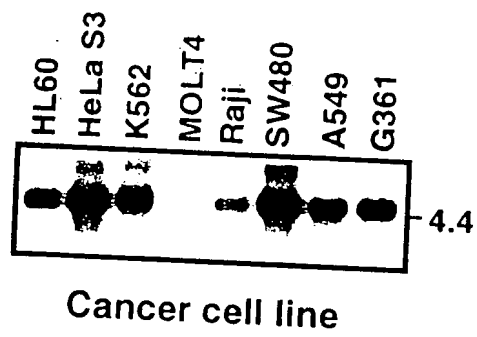


Fig. 6B

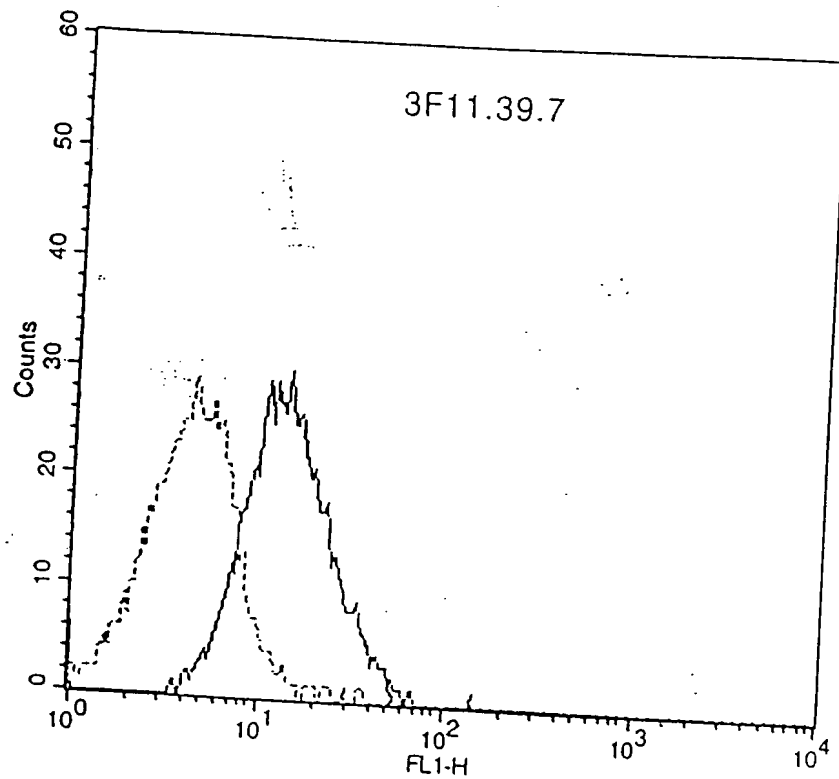


Fig. 7

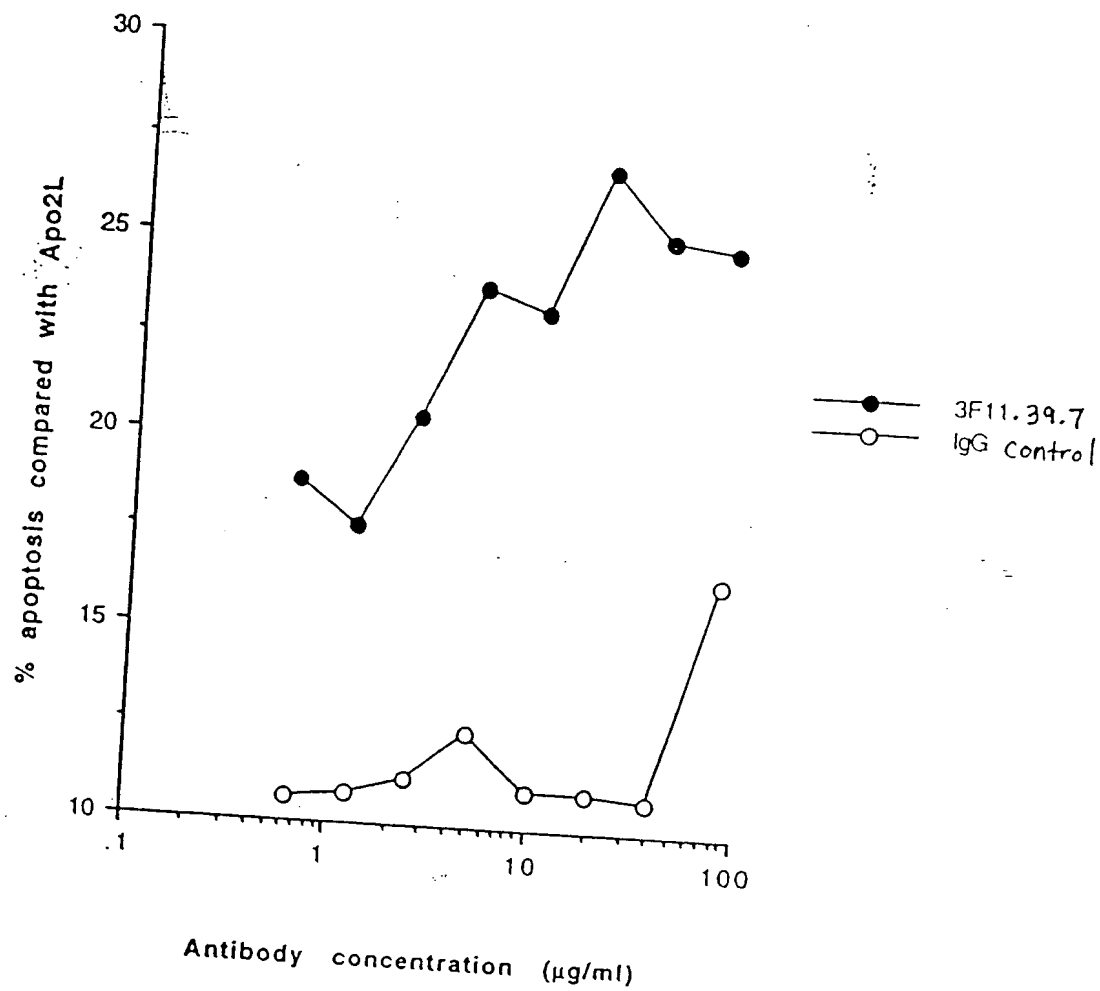


Fig. 8

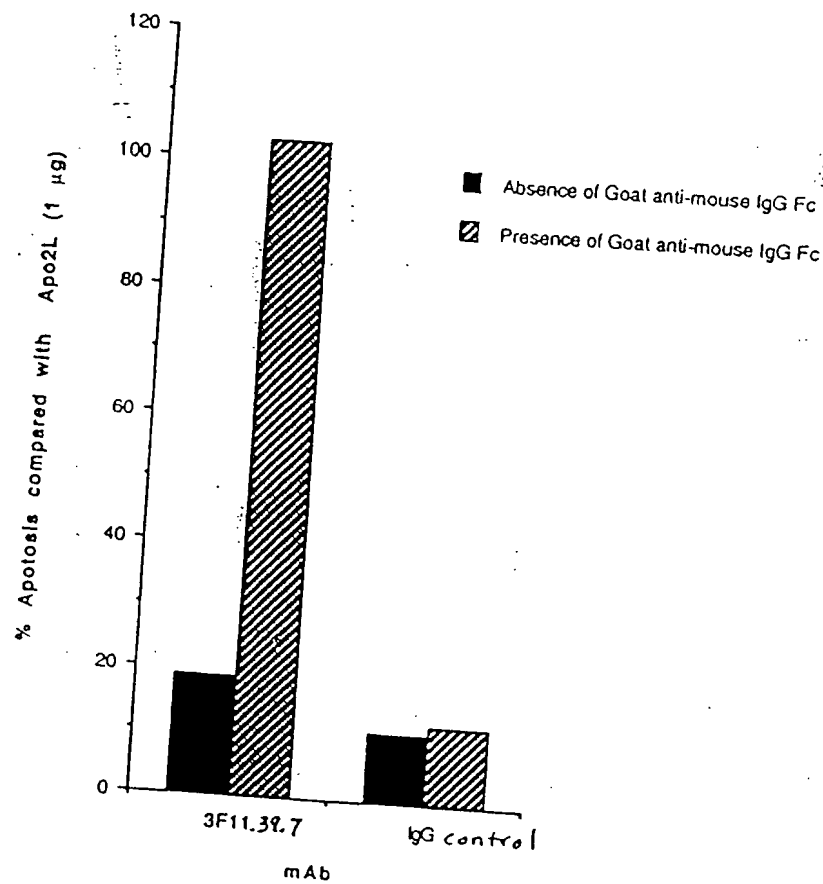


Fig. 9



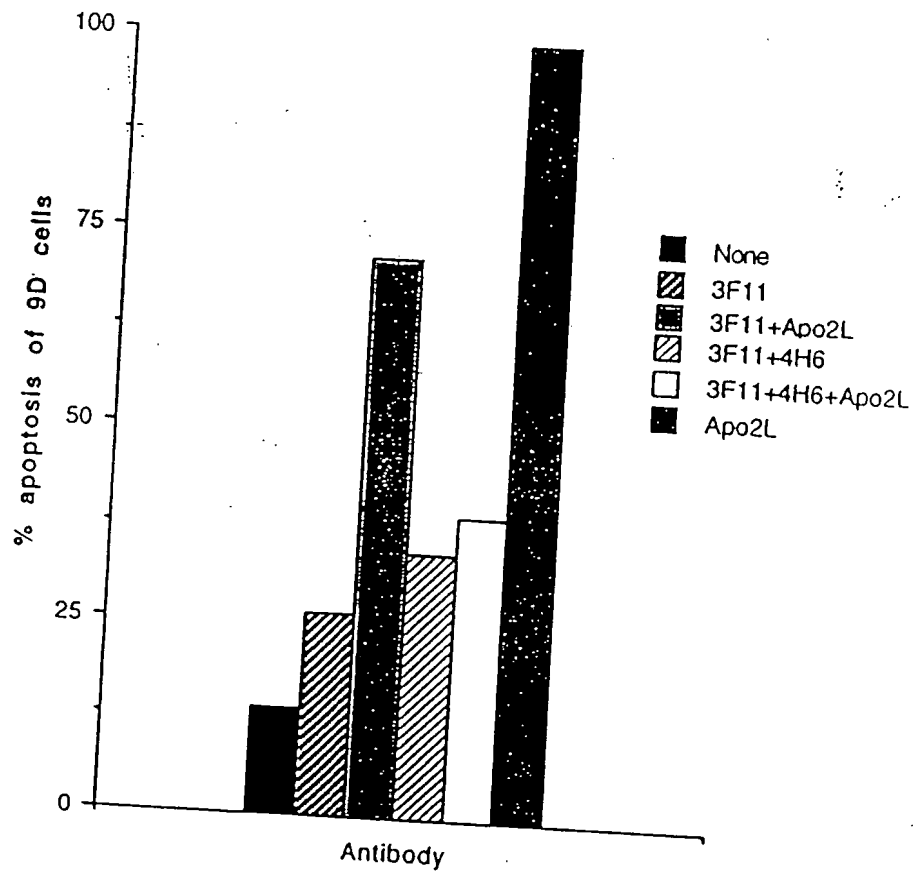


Fig : 10

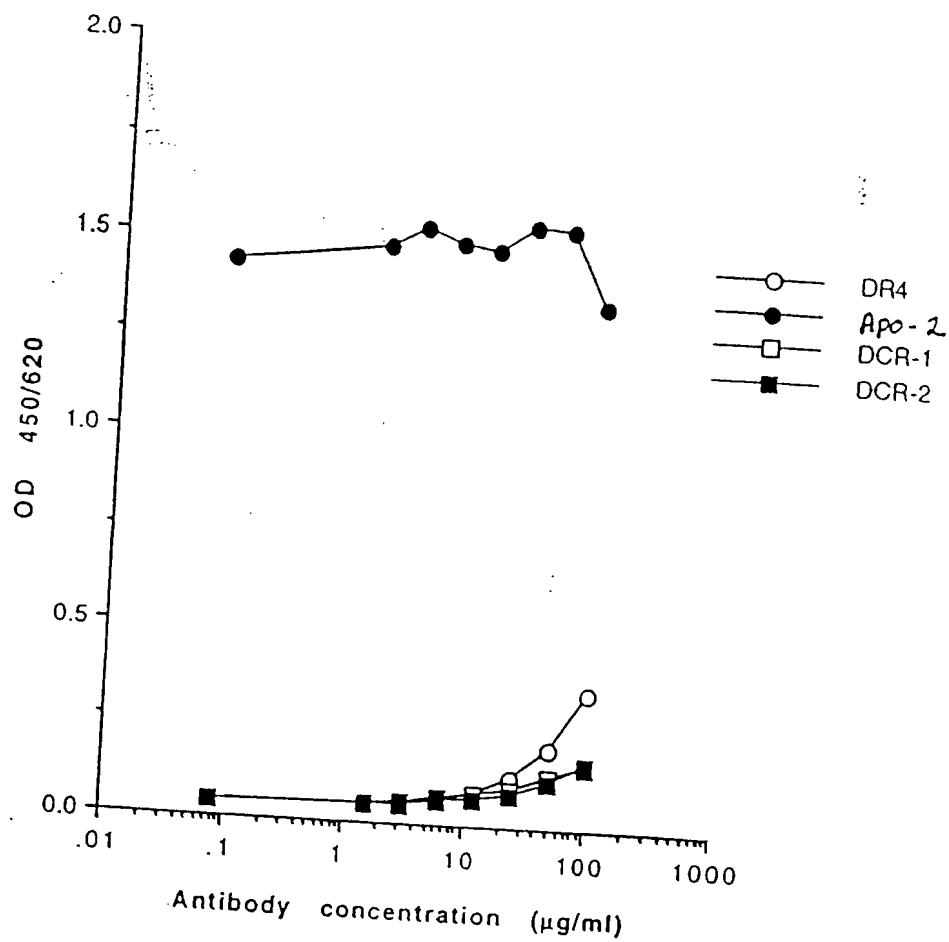


Fig. 11

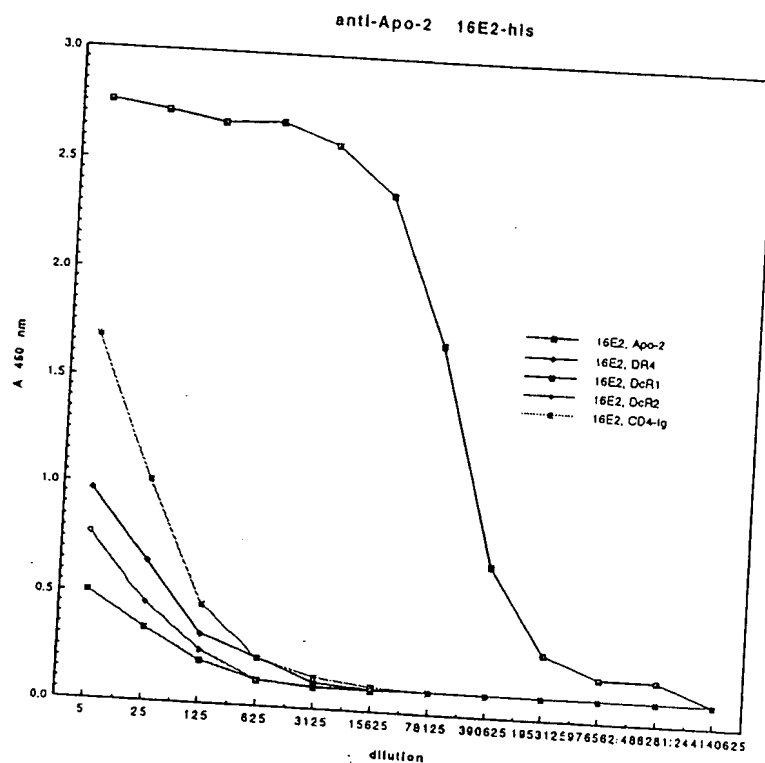


Fig. 12A

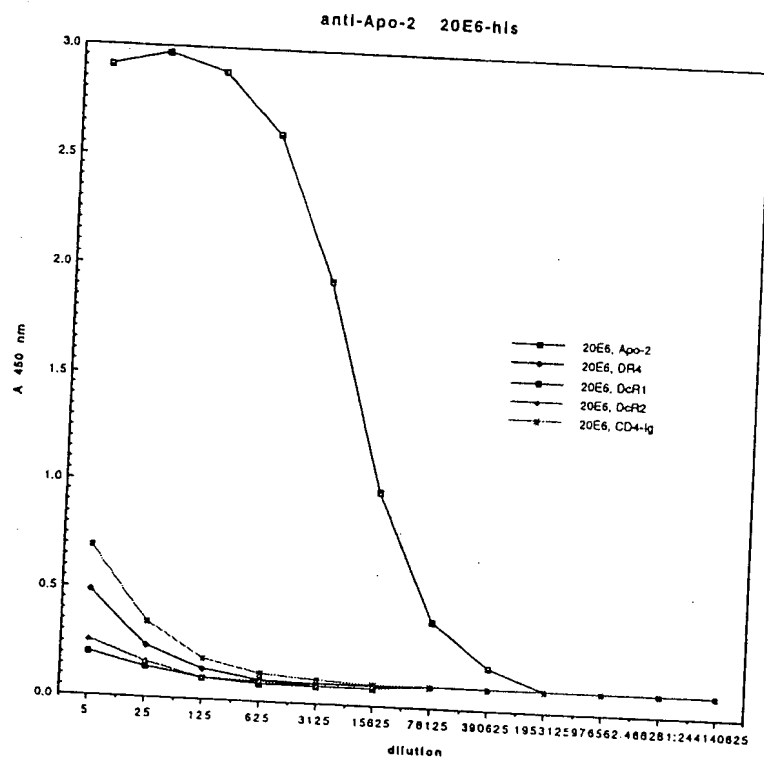


Fig. 12B

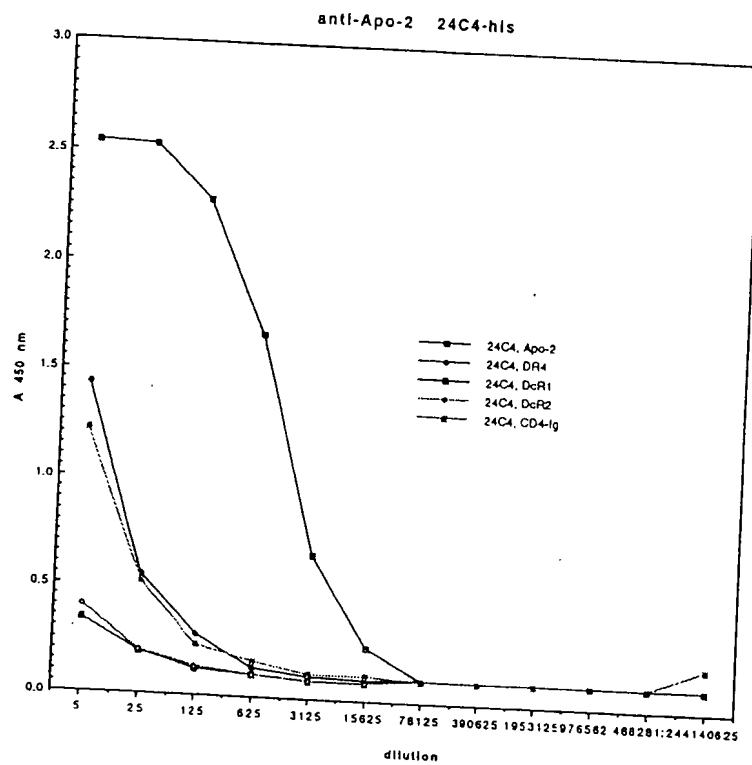


Fig. 12c

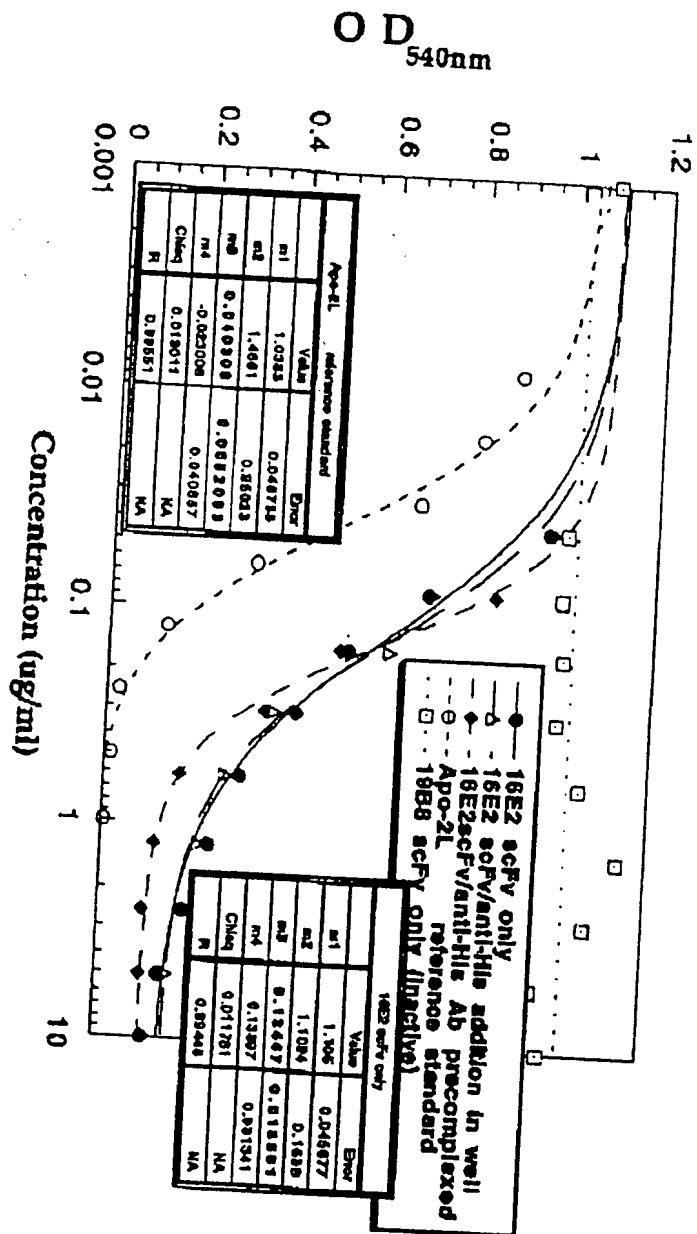


Fig. 13A

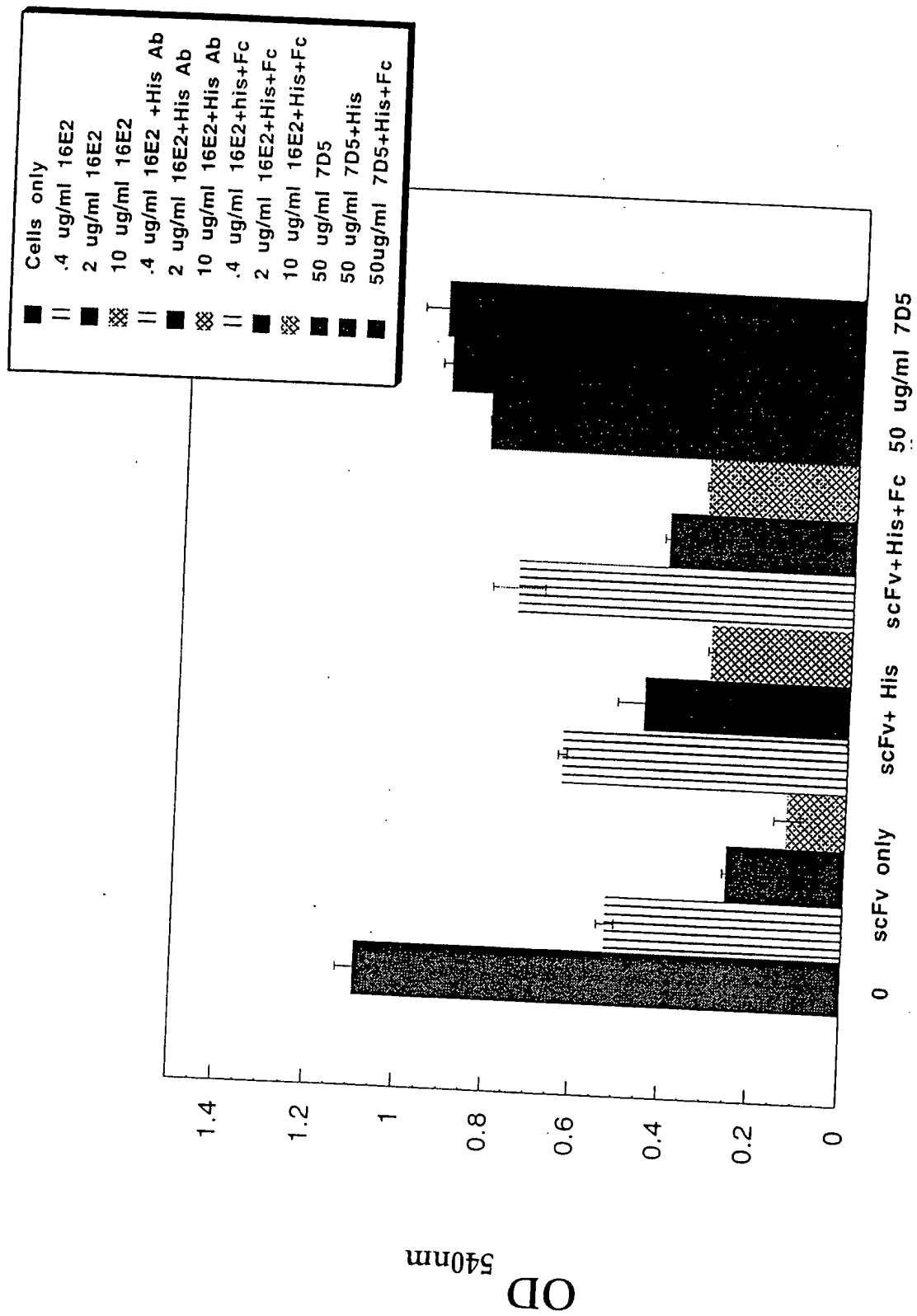


Fig. 13B

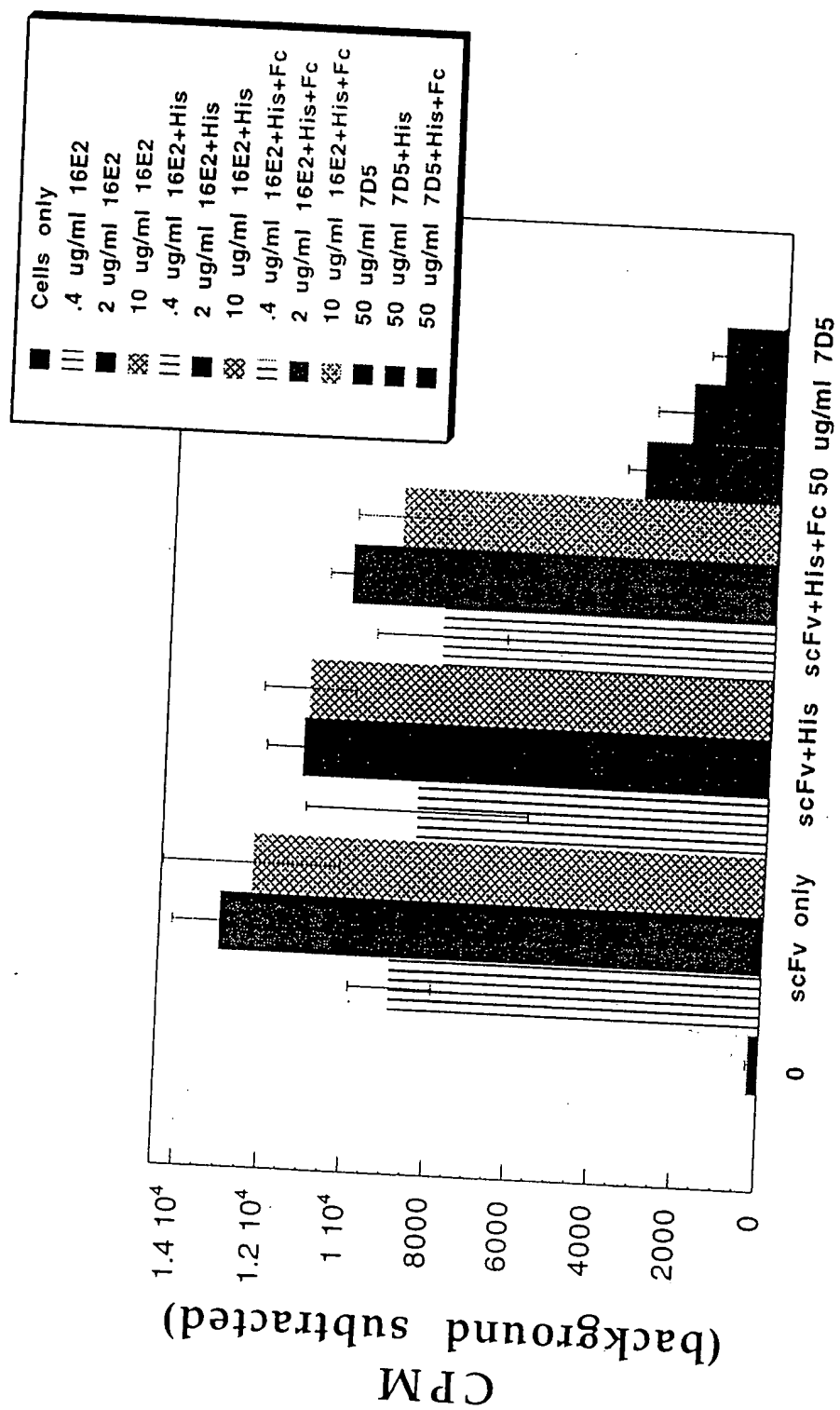


Fig. 13c



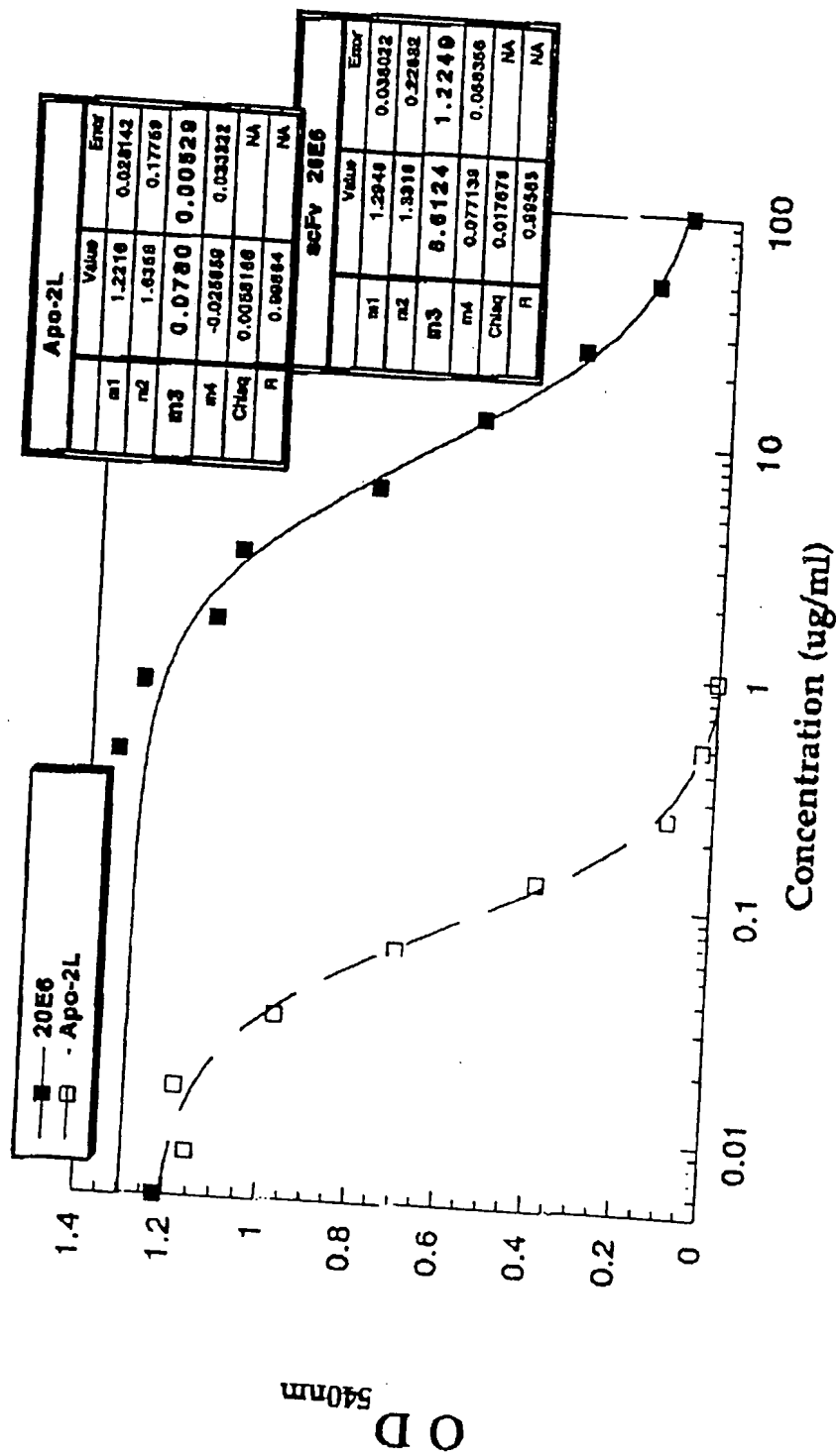


Fig. 14A

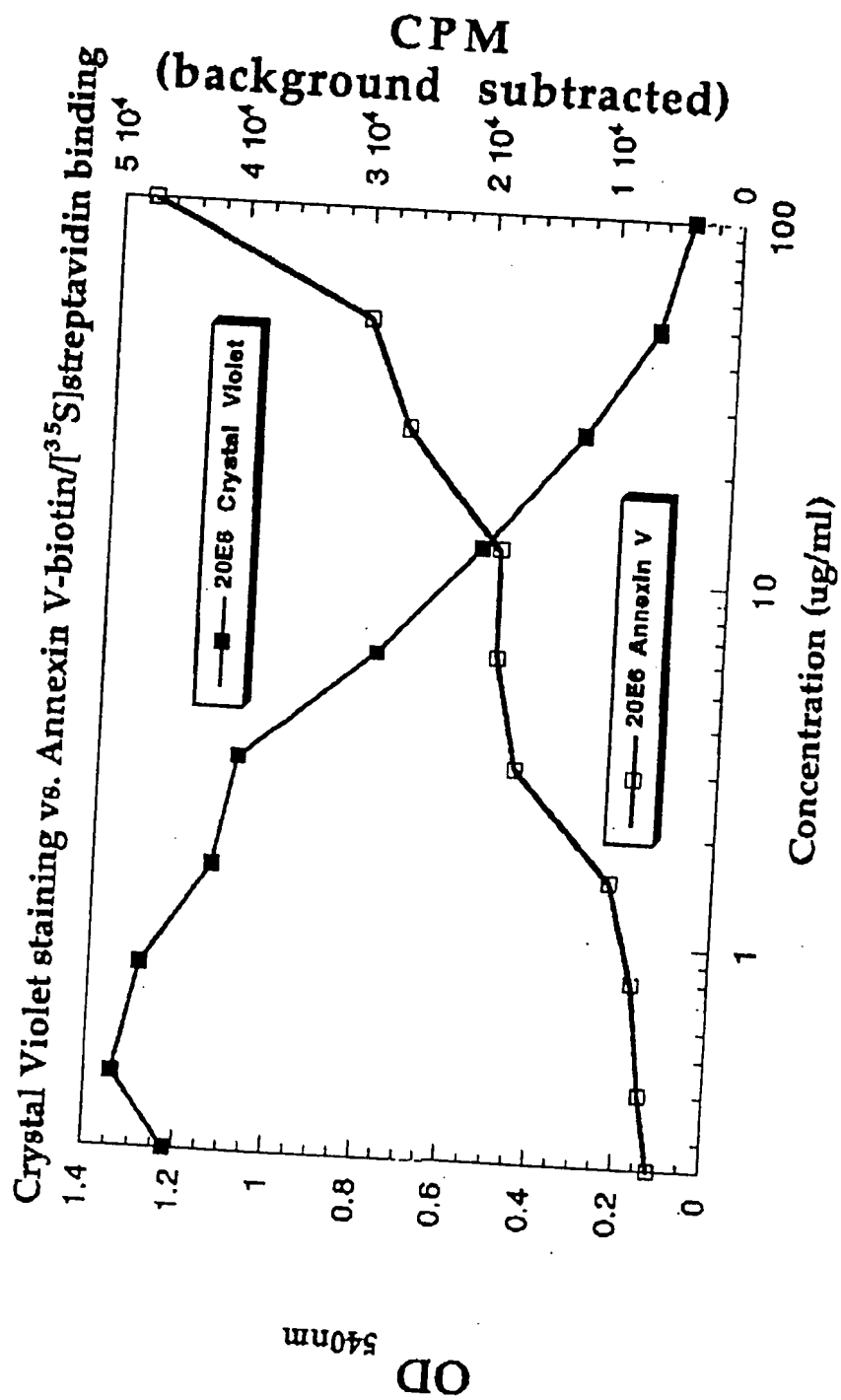


Fig. 14B

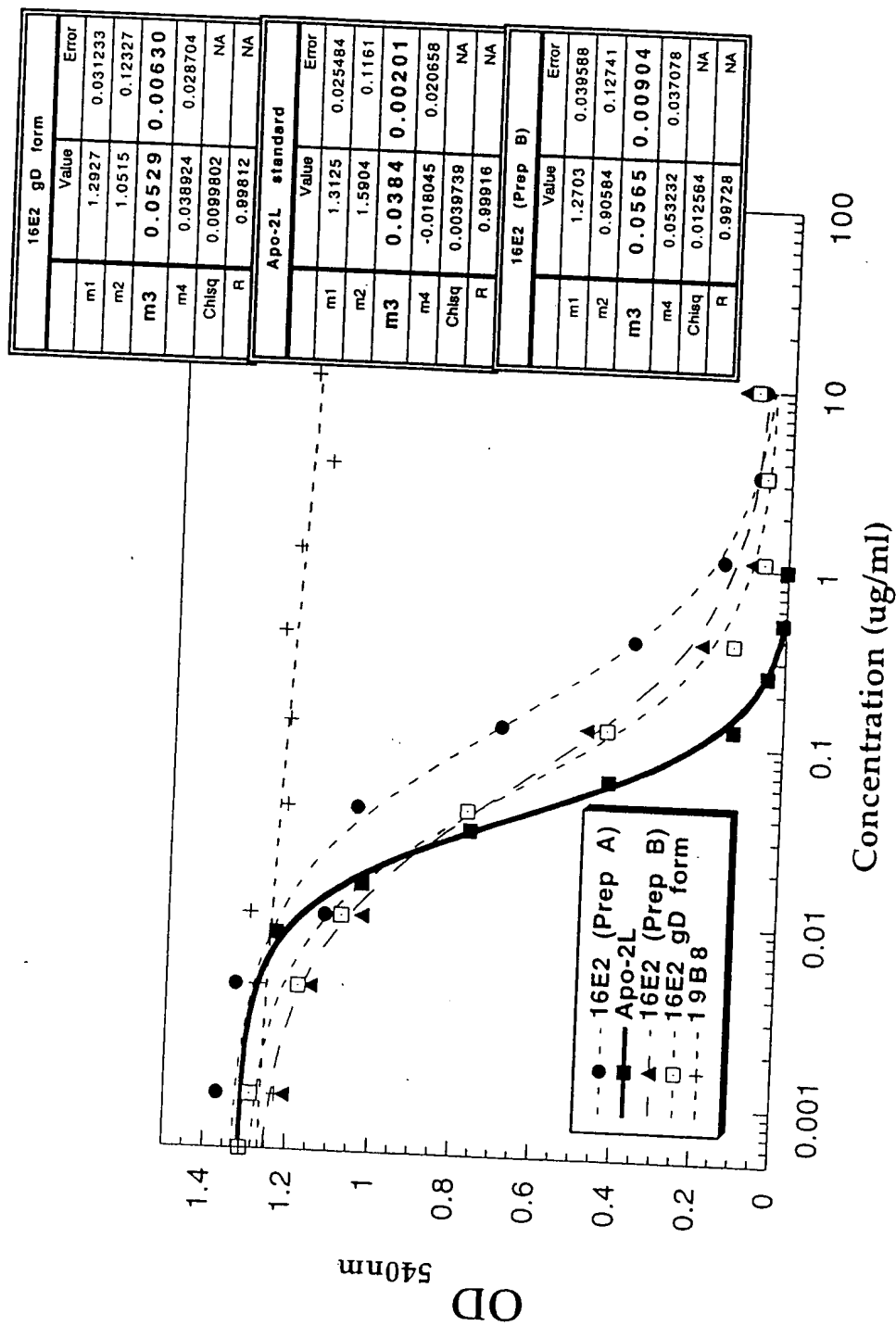


Fig. 14C

ATGACCATGA TTACGCCAAG CTTTGGAGCC TTTTTTTTGG AGATTTTCAA 50  
 CGTGAAAAAA TTATTATTCG CAATTCCTTT AGTTGTCCT TTCTATGCGG 100  
 CCCAGCCGGC CATGGCCGAG GTGCAGCTGG TGCAGTCTGG GGGAGGTGTG 150  
 GAACGGCCCG GGGGGTCCCT GAGACTCTCC TGTGCAGCCT CTGGATTAC 200  
 CTTTGATGAT TATGGCATGA GCTGGGTCCG CCAAGCTCCA GGGAAGGGGC 250  
 TGGAGTGGGT CTCTGGTATT AATTGGAATG GTGGTAGCAC AGGATATGCA 300  
 GACTCTGTGA AGGGCCGAGT CACCATCTCC AGAGACAACG CCAAGAACTC 350  
 CCTGTATCTG CAAATGAACA GCCTGAGAGC CGAGGACACG GCCGTATATT 400  
 ACTGTGCGAA AATCCTGGGT GCCGGAAGGG GCTGGTACTT CGATCTCTGG 450  
 GGGAAGGGGA CCACGGTCAC CGTCTCGAGT GGTGGAGGCG GTTCAGGCGG 500  
 AGGTGGCAGC GGCGGTGGCG GATCGTCTGA GCTGACTCAG GACCCTGCTG 550  
 TGTCTGTGGC CTTGGGACAG ACAGTCAGGA TCACATGCCA AGGAGACAGC 600  
 CTCAGAAGCT ATTATGCAAG CTGGTACCAG CAGAAGCCAG GACAGGCCCC 650  
 TGTACTTGTC ATCTATGGTA AAAACAACCG GCCCTCAGGG ATCCCAGACC 700  
 GATTCTCTGG CTCCAGCTCA GGAAACACAG CTTCTTGAC CATCACTGGG 750  
 GCTCAGGCGG AAGATGAGGC TGA CTATTAC TGTA ACTCCC GGGACAGCAG 800  
 TG GTAACCAT GTGGTATTCG GCGGAGGGAC CAAGCTGACC GTCCTAGGTG 850  
 CGGCCGCACA TCATCATCAC CATCACGGGG CCGCAGAACA AAAACTCATC 900  
 TCAGAAGAGG ATCTGAATGG GGCCGCATAG 930

Fig. 15A

ATGACCATGA TTACGCCAAG CTTTGGAGCC TTTTTTTTGG AGATTTTCAA 50  
 CGTGAAAAAA TTATTATTCG CAATTCCTTT AGTTGTCCTT TTCTATGCGG 100  
 CCCAGCCGGC CATGGCCGGG GTGCAGCTGG TGGAGTCTGG GGGAGGCTTG 150  
 GTCCAGCCTG GGGGGTCCCT GAGACTCTCC TGTGCAGCCT CTGGATTCAC 200  
 CTTTAGTAGC TATTGGATGA GCTGGGTCCG CCAGGCTCCA GGGAAGGGGC 250  
 TGGAGTGGGT GGCCAACATA AAGCAAGATG GAAGTGAGAA ATACTATGTG 300  
 GACTCTGTGA AGGGCCGATT CACCATCTCC AGAGACAACG CCAAGAACTC 350  
 ACTGTATCTG CAAATGAACA GCCTGAGAGC CGAGGACACG GCTGTGTATT 400  
 ACTGTGCGAG AGATCTTTTA AAGGTCAAGG GCAGCTCGTC TGGGTGGTTC 450  
 GACCCCTGGG GGAGAGGGAC CACGGTCACC GTCTCGAGTG GTGGAGGCGG 500  
 TTCAGGCGGA GGTGGTAGCG GCGGTGGCGG ATCGTCTGAG CTGACTCAGG 550  
 ACCCTGCTGT GTCTGTGGCC TTGGGACAGA CAGTCAGGAT CACATGCCAA 600  
 GGAGACAGCC TCAGAAGCTA TTATGCAAGC TGGTACCAGC AGAAGCCAGG 650  
 ACAGGCCCTT GTACTTGTCA TCTATGGTAA AAACAACCGG CCCTCAGGGA 700  
 TCCCAGACCG ATTCTCTGGC TCCAGCTCAG GAAACACAGC TTCCTTGACC 750  
 ATCACTGGGG CTCAGGCGGA AGATGAGGCT GACTATTACT GTAACCTCCG 800  
 GGACAGCAGT GGTAACCATG TGGTATTCCG CGGAGGGACC AAGCTGACCG 850  
 TCCTAGGTGC GGCCGCACAT CATCATCACC ATCACGGGGC CGCAGAACAA 900  
 AAACATCATCT CAGAAGAGGA TCTGAATGGG GCCGCATAG 939

Fig. 15B

ATGACCATGA TTACGCCAAG CTTTGGAGCC TTTT TTTTGG AGATTTTCAA 50  
CGTGAAAAAA TTATTATTCTG CAATTCCTTT AGTTGTTCTT TTCTATGCGG 100  
CCCAGCCGGC CATGGCCCCAG GTGCAGCTGG TGCAGTCTGG GGGAGGCGTG 150  
GTCCAGCCTG GCGGTCCCT GAGACTCTCC TGTGCAGCTT CTGGGTTCAT 200  
TTTCAGTAGT TATGGGATGC ACTGGGTCCG CCAGGCTCCA GGCAAGGGGC 250  
TGGAGTGGGT GGCAGGTATT TTTTATGATG GAGGTAATAA ATACTATGCA 300  
GACTCCGTGA AGGGCCGATT CACCATCTCC AGAGACAATT CCAAGAACAC 350  
GCTGTATCTG CAAATGAACA GCCTGAGAGC TGAGGACACG GCTGTGTATT 400  
ACTGTGCGAG AGATAGGGGC TACTACTACA TGGACGTCTG GGGCAAAGGG 450  
ACCACGGTCA CCGTCTCCTC AGGTGGAGGC GGTTCAGGCG GAGGTGGCTC 500  
TGGCGGTGGC GGATCGCAGT CTGTGTTGAC GCAGCCGCCC TCAGTGTCTG 550  
GGGCCCCAGG ACAGAGGGTC ACCATCTCCT GCACTGGGAG AAGCTCCAAC 600  
ATCGGGGCAG GTCATGATGT ACACTGGTAC CAGCAACTTC CAGGAACAGC 650  
CCCCAACTC CTCATCTATG ATGACAGCAA TCGGCCCTCA GGGGTCCCTG 700  
ACCGATTCTC TGGCTCCAGG TCTGGCACCT CAGCCTCCCT GGCCATCACT 750  
GGGCTCCAGG CTGAAGATGA GGCTGATTAT TACTGCCAGT CCTATGACAG 800  
CAGCCTGAGG GGTTCGGTAT TCGGCGGAGG GACCAAGGTC ACTGTCCTAG 850  
GTGCGGCCGC ACATCATCAT CACCATCACG GGGCCGCAGA AAAAAACTC 900  
ATCTCAGAAG AGGATCTGAA TGGGGCCGCA TAG 933

Fig. 15C

	signal	
Apo-2.16E2.his		Heavy chain
Apo-2.20E6.his	1 MIMITPSFGAFFLEIFNVKKLLFAIPLVVPFYAAQPAMAEVQLVQSGGGV	
Apo-2.24C4.his	1 MIMITPSFGAFFLEIFNVKKLLFAIPLVVPFYAAQPAMAGVQLVESGGGL	
	1 MIMITPSFGAFFLEIFNVKKLLFAIPLVVPFYAAQPAMAQVQLVQSGGGV	
	CDR1	CDR2
Apo-2.16E2.his	51 ERPGGSLRLSCAASGFTTFDDYGM <del>SWRQAPGKGLEWVSGINWNGGSTGYA</del>	
Apo-2.20E6.his	51 VQPGGSLRLSCAASGFTFSSYWM <del>SWRQAPGKGLEWVANIKODGSEKYYV</del>	
Apo-2.24C4.his	51 VQGRSLRLSCAASGFTFSSYGMH <del>SWRQAPGKGLEWVAGIFYDGGNKYYA</del>	
		CDR3
Apo-2.16E2.his	101 DSVKGRVTISRDNAKNSLYLQMN <del>SLRAEDTAVYYCAK IL----</del>	GAGRGWY
Apo-2.20E6.his	101 DSVKGRFTISRDNAKNSLYLQMN <del>SLRAEDTAVYYCARDLLKVKGSSSGW-</del>	
Apo-2.24C4.his	101 DSVKGRFTISRDN <del>SKNTLYLQMN</del> SLRAEDTAVYYCARD-----RGYY	
		Light chain
Apo-2.16E2.his	147 F-DLWGKGTIVTVSSGGGGSGGGGSGGGGS-SELTQDPAVSVALGQTVRI	
Apo-2.20E6.his	150 F-DPWGRGTTIVTVSSGGGGSGGGGSGGGGS-SELTQDPAVSVALGQTVRI	
Apo-2.24C4.his	143 YMDVWGKGTIVTVSSGGGGSGGGGSGGGGSQSVLTQPPSVSGAPGQRVTI	
	CDR1	CDR2
Apo-2.16E2.his	195 TC <del>OGDSL</del> R---SY <del>YASWY</del> QQKPGQAPVLVTY <del>GKNNR</del> PSGIPDRFSGSSSG	
Apo-2.20E6.his	198 TC <del>OGDSL</del> R---SY <del>YASWY</del> QQKPGQAPVLVTY <del>GKNNR</del> PSGIPDRFSGSSSG	
Apo-2.24C4.his	193 SCT <del>GRSSNIGAGHDVH</del> WYQQLPGTAPKLLTY <del>DDSNR</del> PSGVPDRFSGSRSG	
		CDR3
Apo-2.16E2.his	242 NTASLTITGAQAED <del>EADYYCNSRDSSGNHVV</del> FGGGTKLTVLGAAAHHHHH	
Apo-2.20E6.his	245 NTASLTITGAQAED <del>EADYYCNSRDSSGNHVV</del> FGGGTKLTVLGAAAHHHHH	
Apo-2.24C4.his	243 TSASLAITGLQAED <del>EADYYCQSYDSSLRGSV</del> FGGGTKVTVLGAAAHHHHH	
Apo-2.16E2.his	292 HGAAEQKLISEEDLN <del>GAA</del>	
Apo-2.20E6.his	295 HGAAEQKLISEEDLN <del>GAA</del>	
Apo-2.24C4.his	293 HGAAEQKLISEEDLN <del>GAA</del>	

Fig. 16

5

Apo-2 ReceptorRELATED APPLICATIONS

10 This application is a non-provisional application  
claiming priority under Section 119(e) to provisional application  
number 60/046,615 filed May 15, 1997 and provisional application  
number 60/074,119 filed February 9, 1998, the contents of which are  
hereby incorporated by reference.

15

FIELD OF THE INVENTION

The present invention relates generally to the  
identification, isolation, and recombinant production of novel  
polypeptides, designated herein as Apo-2, and to anti-Apo-2  
20 antibodies.

BACKGROUND OF THE INVENTIONApoptosis or "Programmed Cell Death"

Control of cell numbers in mammals is believed to be  
25 determined, in part, by a balance between cell proliferation and  
cell death. One form of cell death, sometimes referred to as  
necrotic cell death, is typically characterized as a pathologic  
form of cell death resulting from some trauma or cellular injury.  
In contrast, there is another, "physiologic" form of cell death  
30 which usually proceeds in an orderly or controlled manner. This  
orderly or controlled form of cell death is often referred to as  
"apoptosis" [see, e.g., Barr et al., Bio/Technology, 12:487-493  
(1994); Steller et al., Science, 267:1445-1449 (1995)]. Apoptotic  
cell death naturally occurs in many physiological processes,  
35 including embryonic development and clonal selection in the immune  
system [Itoh et al., Cell, 66:233-243 (1991)]. Decreased levels of  
apoptotic cell death have been associated with a variety of  
pathological conditions, including cancer, lupus, and herpes virus  
infection [Thompson, Science, 267:1456-1462 (1995)]. Increased



levels of apoptotic cell death may be associated with a variety of other pathological conditions, including AIDS, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, multiple sclerosis, retinitis pigmentosa, cerebellar degeneration, aplastic anemia, myocardial infarction, stroke, reperfusion injury, and toxin-induced liver disease [see, Thompson, supra].

Apoptotic cell death is typically accompanied by one or more characteristic morphological and biochemical changes in cells, such as condensation of cytoplasm, loss of plasma membrane microvilli, segmentation of the nucleus, degradation of chromosomal DNA or loss of mitochondrial function. A variety of extrinsic and intrinsic signals are believed to trigger or induce such morphological and biochemical cellular changes [Raff, Nature, 356:397-400 (1992); Steller, supra; Sachs et al., Blood, 82:15 (1993)]. For instance, they can be triggered by hormonal stimuli, such as glucocorticoid hormones for immature thymocytes, as well as withdrawal of certain growth factors [Watanabe-Fukunaga et al., Nature, 356:314-317 (1992)]. Also, some identified oncogenes such as *myc*, *rel*, and *E1A*, and tumor suppressors, like *p53*, have been reported to have a role in inducing apoptosis. Certain chemotherapy drugs and some forms of radiation have likewise been observed to have apoptosis-inducing activity [Thompson, supra].

#### TNF Family of Cytokines

Various molecules, such as tumor necrosis factor- $\alpha$  ("TNF- $\alpha$ "), tumor necrosis factor- $\beta$  ("TNF- $\beta$ " or "lymphotoxin"), CD30 ligand, CD27 ligand, CD40 ligand, OX-40 ligand, 4-1BB ligand, Apo-1 ligand (also referred to as Fas ligand or CD95 ligand), and Apo-2 ligand (also referred to as TRAIL) have been identified as members of the tumor necrosis factor ("TNF") family of cytokines [See, e.g., Gruss and Dower, Blood, 85:3378-3404 (1995); Wiley et al., Immunity, 3:673-682 (1995); Pitti et al., J. Biol. Chem., 271:12687-12690 (1996); WO 97/01633 published January 16, 1997]. Among these molecules, TNF- $\alpha$ , TNF- $\beta$ , CD30 ligand, 4-1BB ligand, Apo-1 ligand, and Apo-2 ligand (TRAIL) have been reported to be involved in apoptotic cell death. Both TNF- $\alpha$  and TNF- $\beta$  have been reported to induce apoptotic death in susceptible tumor cells [Schmid et al., Proc. Natl. Acad. Sci., 83:1881 (1986); Dealtry et

al., Eur. J. Immunol., 17:689 (1987)]'. Zheng et al. have reported that TNF- $\alpha$  is involved in post-stimulation apoptosis of CD8-positive T cells [Zheng et al., Nature, 377:348-351 (1995)]. Other investigators have reported that CD30 ligand may be involved in deletion of self-reactive T cells in the thymus [Amakawa et al., Cold Spring Harbor Laboratory Symposium on Programmed Cell Death, Abstr. No. 10, (1995)].

Mutations in the mouse Fas/Apo-1 receptor or ligand genes (called *lpr* and *gld*, respectively) have been associated with some autoimmune disorders, indicating that Apo-1 ligand may play a role in regulating the clonal deletion of self-reactive lymphocytes in the periphery [Krammer et al., Curr. Op. Immunol., 6:279-289 (1994); Nagata et al., Science, 267:1449-1456 (1995)]. Apo-1 ligand is also reported to induce post-stimulation apoptosis in CD4-positive T lymphocytes and in B lymphocytes, and may be involved in the elimination of activated lymphocytes when their function is no longer needed [Krammer et al., supra; Nagata et al., supra]. Agonist mouse monoclonal antibodies specifically binding to the Apo-1 receptor have been reported to exhibit cell killing activity that is comparable to or similar to that of TNF- $\alpha$  [Yonehara et al., J. Exp. Med., 169:1747-1756 (1989)].

#### TNF Family of Receptors

Induction of various cellular responses mediated by such TNF family cytokines is believed to be initiated by their binding to specific cell receptors. Two distinct TNF receptors of approximately 55-kDa (TNFR1) and 75-kDa (TNFR2) have been identified [Hohman et al., J. Biol. Chem., 264:14927-14934 (1989); Brockhaus et al., Proc. Natl. Acad. Sci., 87:3127-3131 (1990); EP 417,563, published March 20, 1991] and human and mouse cDNAs corresponding to both receptor types have been isolated and characterized [Loetscher et al., Cell, 61:351 (1990); Schall et al., Cell, 61:361 (1990); Smith et al., Science, 248:1019-1023 (1990); Lewis et al., Proc. Natl. Acad. Sci., 88:2830-2834 (1991); Goodwin et al., Mol. Cell. Biol., 11:3020-3026 (1991)]. Extensive polymorphisms have been associated with both TNF receptor genes [see, e.g., Takao et al., Immunogenetics, 37:199-203 (1993)]. Both TNFRs share the typical structure of cell surface receptors

including extracellular, transmembrane and intracellular regions. The extracellular portions of both receptors are found naturally also as soluble TNF-binding proteins [Nophar, Y. et al., EMBO J., 9:3269 (1990); and Kohno, T. et al., Proc. Natl. Acad. Sci. U.S.A., 87:8331 (1990)]. The cloning of recombinant soluble TNF receptors was reported by Hale et al. [J. Cell. Biochem. Supplement 15F, 1991, p. 113 (P424)].

The extracellular portion of type 1 and type 2 TNFRs (TNFR1 and TNFR2) contains a repetitive amino acid sequence pattern of four cysteine-rich domains (CRDs) designated 1 through 4, starting from the NH<sub>2</sub>-terminus. Each CRD is about 40 amino acids long and contains 4 to 6 cysteine residues at positions which are well conserved [Schall et al., supra; Loetscher et al., supra; Smith et al., supra; Nophar et al., supra; Kohno et al., supra]. In TNFR1, the approximate boundaries of the four CRDs are as follows: CRD1- amino acids 14 to about 53; CRD2- amino acids from about 54 to about 97; CRD3- amino acids from about 98 to about 138; CRD4- amino acids from about 139 to about 167. In TNFR2, CRD1 includes amino acids 17 to about 54; CRD2- amino acids from about 55 to about 97; CRD3- amino acids from about 98 to about 140; and CRD4- amino acids from about 141 to about 179 [Banner et al., Cell, 73:431-435 (1993)]. The potential role of the CRDs in ligand binding is also described by Banner et al., supra.

A similar repetitive pattern of CRDs exists in several other cell-surface proteins, including the p75 nerve growth factor receptor (NGFR) [Johnson et al., Cell, 47:545 (1986); Radeke et al., Nature, 325:593 (1987)], the B cell antigen CD40 [Stamenkovic et al., EMBO J., 8:1403 (1989)], the T cell antigen OX40 [Mallet et al., EMBO J., 9:1063 (1990)] and the Fas antigen [Yonehara et al., supra and Itoh et al., supra]. CRDs are also found in the soluble TNFR (sTNFR)-like T2 proteins of the Shope and myxoma poxviruses [Upton et al., Virology, 160:20-29 (1987); Smith et al., Biochem. Biophys. Res. Commun., 176:335 (1991); Upton et al., Virology, 184:370 (1991)]. Optimal alignment of these sequences indicates that the positions of the cysteine residues are well conserved. These receptors are sometimes collectively referred to as members of the TNF/NGF receptor superfamily. Recent studies on p75NGFR showed that the deletion of CRD1 [Welcher, A.A. et al., Proc. Natl. Acad. Sci. USA, 88:159-163 (1991)] or a 5-amino acid insertion in

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this domain [Yan, H. and Chao, M.V., J. Biol. Chem., 266:12099-12104 (1991)] had little or no effect on NGF binding [Yan, H. and Chao, M.V., supra]. p75 NGFR contains a proline-rich stretch of about 60 amino acids, between its CRD4 and transmembrane region, which is not involved in NGF binding [Peetre, C. et al., Eur. J. Hematol., 41:414-419 (1988); Seckinger, P. et al., J. Biol. Chem., 264:11966-11973 (1989); Yan, H. and Chao, M.V., supra]. A similar proline-rich region is found in TNFR2 but not in TNFR1.

Itoh et al. disclose that the Apo-1 receptor can signal an apoptotic cell death similar to that signaled by the 55-kDa TNFR1 [Itoh et al., supra]. Expression of the Apo-1 antigen has also been reported to be down-regulated along with that of TNFR1 when cells are treated with either TNF- $\alpha$  or anti-Apo-1 mouse monoclonal antibody [Krammer et al., supra; Nagata et al., supra]. Accordingly, some investigators have hypothesized that cell lines that co-express both Apo-1 and TNFR1 receptors may mediate cell killing through common signaling pathways [Id.].

The TNF family ligands identified to date, with the exception of lymphotoxin- $\alpha$ , are type II transmembrane proteins, whose C-terminus is extracellular. In contrast, the receptors in the TNF receptor (TNFR) family identified to date are type I transmembrane proteins. In both the TNF ligand and receptor families, however, homology identified between family members has been found mainly in the extracellular domain ("ECD"). Several of the TNF family cytokines, including TNF- $\alpha$ , Apo-1 ligand and CD40 ligand, are cleaved proteolytically at the cell surface; the resulting protein in each case typically forms a homotrimeric molecule that functions as a soluble cytokine. TNF receptor family proteins are also usually cleaved proteolytically to release soluble receptor ECDs that can function as inhibitors of the cognate cytokines.

Recently, other members of the mammalian TNFR family have been identified. In Marsters et al., Curr. Biol., 6:750 (1996), investigators describe a full length native sequence human polypeptide, called Apo-3, which exhibits similarity to the TNFR family in its extracellular cysteine-rich repeats and resembles TNFR1 and CD95 in that it contains a cytoplasmic death domain sequence [see also Marsters et al., Curr. Biol., 6:1669 (1996)].

FIGURE 2

Apo-3 has also been referred to by other investigators as DR3, wsl-1 and TRAMP [Chinnaiyan et al., Science, 274:990 (1996); Kitson et al., Nature, 384:372 (1996); Bodmer et al., Immunity, 6:79 (1997)].

Pan et al. have disclosed another TNF receptor family member referred to as "DR4" [Pan et al., Science, 276:111-113 (1997)]. The DR4 was reported to contain a cytoplasmic death domain capable of engaging the cell suicide apparatus. Pan et al. disclose that DR4 is believed to be a receptor for the ligand known as Apo-2 ligand or TRAIL.

#### The Apoptosis-Inducing Signaling Complex

As presently understood, the cell death program contains at least three important elements - activators, inhibitors, and effectors; in *C. elegans*, these elements are encoded respectively by three genes, *Ced-4*, *Ced-9* and *Ced-3* [Steller, Science, 267:1445 (1995); Chinnaiyan et al., Science, 275:1122-1126 (1997)]. Two of the TNFR family members, TNFR1 and Fas/Apo1 (CD95), can activate apoptotic cell death [Chinnaiyan and Dixit, Current Biology, 6:555-562 (1996); Fraser and Evan, Cell, 85:781-784 (1996)]. TNFR1 is also known to mediate activation of the transcription factor, NF- $\kappa$ B [Tartaglia et al., Cell, 74:845-853 (1993); Hsu et al., Cell, 84:299-308 (1996)]. In addition to some ECD homology, these two receptors share homology in their intracellular domain (ICD) in an oligomerization interface known as the death domain [Tartaglia et al., supra; Nagata, Cell, 88:355 (1997)]. Death domains are also found in several metazoan proteins that regulate apoptosis, namely, the *Drosophila* protein, Reaper, and the mammalian proteins referred to as FADD/MORT1, TRADD, and RIP [Cleaveland and Ihle, Cell, 81:479-482 (1995)]. Using the yeast-two hybrid system, Raven et al. report the identification of protein, wsl-1, which binds to the TNFR1 death domain [Raven et al., Programmed Cell Death Meeting, September 20-24, 1995, Abstract at page 127; Raven et al., European Cytokine Network, 7:Abstr. 82 at page 210 (April-June 1996)]. The wsl-1 protein is described as being homologous to TNFR1 (48% identity) and having a restricted tissue distribution. According to Raven et al., the tissue distribution of wsl-1 is significantly different from the TNFR1 binding protein, TRADD.

Upon ligand binding and receptor clustering, TNFR1 and CD95 are believed to recruit FADD into a death-inducing signalling

complex. CD95 purportedly binds FADD directly, while TNFR1 binds FADD indirectly via TRADD [Chinnaiyan et al., Cell, 81:505-512 (1995); Boldin et al., J. Biol. Chem., 270:387-391 (1995); Hsu et al., supra; Chinnaiyan et al., J. Biol. Chem., 271:4961-4965 (1996)]. It has been reported that FADD serves as an adaptor protein which recruits the Ced-3-related protease, MACH $\alpha$ /FLICE (caspase 8), into the death signalling complex [Boldin et al., Cell, 85:803-815 (1996); Muzio et al., Cell, 85:817-827 (1996)]. MACH $\alpha$ /FLICE appears to be the trigger that sets off a cascade of apoptotic proteases, including the interleukin-1 $\beta$  converting enzyme (ICE) and CPP32/Yama, which may execute some critical aspects of the cell death programme [Fraser and Evan, supra].

It was recently disclosed that programmed cell death involves the activity of members of a family of cysteine proteases related to the *C. elegans* cell death gene, *ced-3*, and to the mammalian IL-1-converting enzyme, ICE. The activity of the ICE and CPP32/Yama proteases can be inhibited by the product of the cowpox virus gene, *crmA* [Ray et al., Cell, 69:597-604 (1992); Tewari et al., Cell, 81:801-809 (1995)]. Recent studies show that CrmA can inhibit TNFR1- and CD95-induced cell death [Enari et al., Nature, 375:78-81 (1995); Tewari et al., J. Biol. Chem., 270:3255-3260 (1995)].

As reviewed recently by Tewari et al., TNFR1, TNFR2 and CD40 modulate the expression of proinflammatory and costimulatory cytokines, cytokine receptors, and cell adhesion molecules through activation of the transcription factor, NF- $\kappa$ B [Tewari et al., Curr. Op. Genet. Develop., 6:39-44 (1996)]. NF- $\kappa$ B is the prototype of a family of dimeric transcription factors whose subunits contain conserved Rel regions [Verma et al., Genes Develop., 9:2723-2735 (1996); Baldwin, Ann. Rev. Immunol., 14:649-681 (1996)]. In its latent form, NF- $\kappa$ B is complexed with members of the I $\kappa$ B inhibitor family; upon inactivation of the I $\kappa$ B in response to certain stimuli, released NF- $\kappa$ B translocates to the nucleus where it binds to specific DNA sequences and activates gene transcription.

For a review of the TNF family of cytokines and their receptors, see Gruss and Dower, supra.

## SUMMARY OF THE INVENTION

Applicants have identified cDNA clones that encode novel polypeptides, designated in the present application as "Apo-2." It is believed that Apo-2 is a member of the TNFR family; full-length native sequence human Apo-2 polypeptide exhibits some similarities to some known TNFRs, including a cytoplasmic death domain region. Full-length native sequence human Apo-2 also exhibits similarity to the TNFR family in its extracellular cysteine-rich repeats. Apo-2 polypeptide has been found to be capable of triggering caspase-dependent apoptosis and activating NF- $\kappa$ B. Applicants surprisingly found that a soluble extracellular domain of Apo-2 binds Apo-2 ligand ("Apo-2L") and can inhibit Apo-2 ligand function. It is presently believed that Apo-2 ligand can signal via at least two different receptors, DR4 and the newly described Apo-2 herein.

In one embodiment, the invention provides isolated Apo-2 polypeptide. In particular, the invention provides isolated native sequence Apo-2 polypeptide, which in one embodiment, includes an amino acid sequence comprising residues 1 to 411 of Figure 1 (SEQ ID NO:1). In other embodiments, the isolated Apo-2 polypeptide comprises at least about 80% amino acid sequence identity with native sequence Apo-2 polypeptide comprising residues 1 to 411 of Figure 1 (SEQ ID NO:1). Optionally, the Apo-2 polypeptide is obtained or obtainable by expressing the polypeptide encoded by the cDNA insert of the vector deposited as ATCC 209021.

In another embodiment, the invention provides an isolated extracellular domain (ECD) sequence of Apo-2. Optionally, the isolated extracellular domain sequence comprises amino acid residues 54 to 182 of Fig. 1 (SEQ ID NO:1).

In another embodiment, the invention provides an isolated death domain sequence of Apo-2. Optionally, the isolated death domain sequence comprises amino acid residues 324 to 391 of Fig. 1 (SEQ ID NO:1).

In another embodiment, the invention provides chimeric molecules comprising Apo-2 polypeptide fused to a heterologous polypeptide or amino acid sequence. An example of such a chimeric molecule comprises an Apo-2 fused to an immunoglobulin sequence. Another example comprises an extracellular domain sequence of Apo-2 fused to a heterologous polypeptide or amino acid sequence, such as an immunoglobulin sequence.

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In another embodiment, the invention provides an isolated nucleic acid molecule encoding Apo-2 polypeptide. In one aspect, the nucleic acid molecule is RNA or DNA that encodes an Apo-2 polypeptide or a particular domain of Apo-2, or is  
5 complementary to such encoding nucleic acid sequence, and remains stably bound to it under at least moderate, and optionally, under high stringency conditions. Such complementary nucleic acid may be fully complementary to the entire length of the RNA or DNA. It is contemplated that the complementary nucleic acid may also be  
10 complementary to only a fragment of the RNA or DNA nucleotide sequence. In one embodiment, the nucleic acid sequence is selected from:

(a) the coding region of the nucleic acid sequence of Figure 1 (SEQ ID NO:2) that codes for residue 1 to residue 411  
15 (i.e., nucleotides 140-142 through 1370-1372), inclusive;

(b) the coding region of the nucleic acid sequence of Figure 1 (SEQ ID NO:2) that codes for residue 1 to residue 182 (i.e., nucleotides 140-142 through 683-685), inclusive;

(c) the coding region of the nucleic acid sequence of  
20 Figure 1 (SEQ ID NO:2) that codes for residue 54 to residue 182 (i.e., nucleotides 299-301 through 683-685), inclusive;

(d) the coding region of the nucleic acid sequence of Figure 1 (SEQ ID NO:2) that codes for residue 324 to residue 391 (i.e., nucleotides 1109-1111 through 1310-1312), inclusive; or

25 (e) a sequence corresponding to the sequence of (a), (b), (c) or (d) within the scope of degeneracy of the genetic code. The isolated nucleic acid may comprise the Apo-2 polypeptide cDNA insert of the vector deposited as ATCC 209021 which includes the nucleotide sequence encoding Apo-2 polypeptide.

30 In a further embodiment, the invention provides a vector comprising the nucleic acid molecule encoding the Apo-2 polypeptide or particular domain of Apo-2. A host cell comprising the vector or the nucleic acid molecule is also provided. A method of producing Apo-2 is further provided.

35 In another embodiment, the invention provides an antibody which specifically binds to Apo-2. The antibody may be an agonistic, antagonistic or neutralizing antibody. Single-chain antibodies and dimeric molecules, in particular homodimeric molecules, comprising Apo-2 antibody are also provided.



In another embodiment, the invention provides non-human, transgenic or knock-out animals.

A further embodiment of the invention provides articles of manufacture and kits that include Apo-2 or Apo-2 antibodies.

5

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the nucleotide sequence of a native sequence human Apo-2 cDNA (SEQ ID NO:2) and its derived amino acid sequence (SEQ ID NO:1).

10

Figure 2A shows the derived amino acid sequence of a native sequence human Apo-2 - the putative signal sequence is underlined, the putative transmembrane domain is boxed, and the putative death domain sequence is dash underlined. The cysteines of the two cysteine-rich domains are individually underlined.

15

Figure 2B shows an alignment and comparison of the death domain sequences of native sequence human Apo-2, DR4, Apo-3/DR3, TNFR1, and Fas/Apo-1 (CD95). Asterisks indicate residues that are essential for death signaling by TNFR1 [Tartaglia et al., *supra*].

20

Figure 3 shows the interaction of the Apo-2 ECD with Apo-2L. Supernatants from mock-transfected 293 cells or from 293 cells transfected with Flag epitope-tagged Apo-2 ECD were incubated with poly-His-tagged Apo-2L and subjected to immunoprecipitation with anti-Flag conjugated or Nickel conjugated agarose beads. The precipitated proteins were resolved by electrophoresis on polyacrylamide gels, and detected by immunoblot with anti-Apo-2L or anti-Flag antibody.

25

Figure 4 shows the induction of apoptosis by Apo-2 and inhibition of Apo-2L activity by soluble Apo-2 ECD. Human 293 cells (A, B) or HeLa cells (C) were transfected by pRK5 vector or by pRK5-based plasmids encoding Apo-2 and/or CrmA. Apoptosis was assessed by morphology (A), DNA fragmentation (B), or by FACS (C-E). Soluble Apo-2L was pre-incubated with buffer or affinity-purified Apo-2 ECD together with anti-Flag antibody or Apo-2 ECD immunoadhesin or DR4 or TNFR1 immunoadhesins and added to HeLa cells. The cells were later analyzed for apoptosis (D). Dose-response analysis using Apo-2L with Apo-2 ECD immunoadhesin was also determined (E).

30

35

Figure 5 shows activation of NF- $\kappa$ B by Apo-2, DR4, and Apo-2L. (A) HeLa cells were transfected with expression plasmids

encoding the indicated proteins. Nuclear extracts were prepared and analyzed by an electrophoretic mobility shift assay. (B) HeLa cells or MCF7 cells were treated with buffer, Apo-2L or TNF-alpha and assayed for NF-kB activity. (C) HeLa cells were preincubated with buffer, ALLN or cyclohexamide before addition of Apo-2L. Apoptosis was later analyzed by FACS.

Figure 6A shows expression of Apo-2 mRNA in human tissues as analyzed by Northern hybridization of human tissue poly A RNA blots.

Figure 6B shows expression of Apo-2 mRNA in human cancer cell lines as analyzed by Northern hybridization of human cancer cell line poly A RNA blots.

Figure 7 shows the FACS analysis of an Apo-2 antibody, 3F11.39.7 (illustrated by the bold lines) as compared to IgG controls (dotted lines). The 3F11.39.7 antibody recognized the Apo-2 receptor expressed in human 9D cells.

Figure 8 is a graph showing percent (%) apoptosis induced in 9D cells by Apo-2 antibody 3F11.39.7, in the absence of goat anti-mouse IgG Fc.

Figure 9 is a bar diagram showing percent (%) apoptosis, as compared to Apo-2L, in 9D cells by Apo-2 antibody 3F11.39.7 in the presence or absence of goat anti-mouse IgG Fc.

Figure 10 is a bar diagram illustrating the ability of Apo-2 antibody 3F11.39.7 to block the apoptosis induced by Apo-2L in 9D cells.

Figure 11 is a graph showing results of an ELISA testing binding of Apo-2 antibody 3F11.39.7 to Apo-2 and to other known Apo-2L receptors referred to as DR4, DcR1, and DcR2.

Figure 12A is a graph showing the results of an ELISA assay evaluating binding of the 16E2 antibody to Apo-2, DR4, DcR1, DcR2 and CD4-Ig.

Figure 12B is a graph showing the results of an ELISA assay evaluating binding of the 20E6 antibody to Apo-2, DR4, DcR1, DcR2 and CD4-Ig.

Figure 12C is a graph showing the results of an ELISA assay evaluating binding of the 24C4 antibody to Apo-2, DR4, DcR1, DcR2 and CD4-Ig.

Figure 13A is a graph showing agonistic activity of the 16E2 antibody, as compared to Apo-2L, in an apoptosis assay (crystal violet stain) using SK-MES-1 cells.

Figure 13B is a bar diagram showing agonistic activity of the 16E2 antibody, as compared to 7D5 scFv antibody (an anti-tissue factor antibody), in an apoptosis assay (crystal violet stain) using SK-MES-1 cells.

Figure 13C is a bar diagram showing agonistic activity of the 16E2 antibody, as compared to 7D5 scFv antibody, in an apoptosis assay (annexin V-biotin/streptavidin-[S<sup>35</sup>]) using SK-MES-1 cells.

Figure 14A is a graph showing agonistic activity of the 20E6 antibody, as compared to Apo-2L, in an apoptosis assay (crystal violet stain) using SK-MES-1 cells.

Figure 14B is a graph showing agonistic activity of the 20E6 antibody by a comparison between results obtained in the crystal violet and annexin V-biotin/streptavidin-[S<sup>35</sup>] apoptosis assays.

Figure 14C is a graph showing agonistic activity of gD-tagged 16E2 antibody, as compared to Apo-2L, in an apoptosis assay (crystal violet stain) using SK-MES-1 cells.

Figure 15A shows the nucleotide sequence of the single chain antibody (scFv) fragment referred to as 16E2 (SEQ ID NO:6).

Figure 15B shows the nucleotide sequence of the single chain antibody (scFv) fragment referred to as 20E6 (SEQ ID NO:7).

Figure 15C shows the nucleotide sequence of the single chain antibody (scFv) fragment referred to as 24C4 (SEQ ID NO:8).

Figure 16 shows the single chain antibody (scFv) fragments referred to as 16E2, 20E6 and 24C4, with the respective amino acid sequences for the signal sequence and the heavy and light chain CDR regions identified (CDR1, CDR2, and CDR3 regions are underlined).

### 35 DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

#### I. Definitions

The terms "Apo-2 polypeptide" and "Apo-2" when used herein encompass native sequence Apo-2 and Apo-2 variants (which

are further defined herein). These terms encompass Apo-2 from a variety of mammals, including humans. The Apo-2 may be isolated from a variety of sources, such as from human tissue types or from another source, or prepared by recombinant or synthetic methods.

5 A "native sequence Apo-2" comprises a polypeptide having the same amino acid sequence as an Apo-2 derived from nature. Thus, a native sequence Apo-2 can have the amino acid sequence of naturally-occurring Apo-2 from any mammal. Such native sequence Apo-2 can be isolated from nature or can be produced by recombinant  
10 or synthetic means. The term "native sequence Apo-2" specifically encompasses naturally-occurring truncated or secreted forms of the Apo-2 (e.g., an extracellular domain sequence), naturally-occurring variant forms (e.g., alternatively spliced forms) and naturally-occurring allelic variants of the Apo-2. A naturally-occurring  
15 variant form of the Apo-2 includes an Apo-2 having an amino acid substitution at residue 410 in the amino acid sequence shown in Figure 1 (SEQ ID NO:1). In one embodiment of such naturally-occurring variant form, the leucine residue at position 410 is substituted by a methionine residue. In Fig. 1 (SEQ ID NO:1), the  
20 amino acid residue at position 410 is identified as "Xaa" to indicate that the amino acid may, optionally, be either leucine or methionine. In Fig. 1 (SEQ ID NO:2), the nucleotide at position 1367 is identified as "W" to indicate that the nucleotide may be either adenine (A) or thymine (T) or uracil (U). In one embodiment  
25 of the invention, the native sequence Apo-2 is a mature or full-length native sequence Apo-2 comprising amino acids 1 to 411 of Fig. 1 (SEQ ID NO:1). Optionally, the Apo-2 is obtained or obtainable by expressing the polypeptide encoded by the cDNA insert of the vector deposited as ATCC 209021.

30 The "Apo-2 extracellular domain" or "Apo-2 ECD" refers to a form of Apo-2 which is essentially free of the transmembrane and cytoplasmic domains of Apo-2. Ordinarily, Apo-2 ECD will have less than 1% of such transmembrane and/or cytoplasmic domains and preferably, will have less than 0.5% of such domains. Optionally,  
35 Apo-2 ECD will comprise amino acid residues 54 to 182 of Fig. 1 (SEQ ID NO:1) or amino acid residues 1 to 182 of Fig. 1 (SEQ ID NO:1). Optionally, Apo-2 ECD will comprise one or more cysteine-rich domains, and preferably, one or both of the cysteine-rich domains identified herein (see Figure 2A). It will be understood

by the skilled artisan that the transmembrane domain identified for the Apo-2 polypeptide herein is identified pursuant to criteria routinely employed in the art for identifying that type of hydrophobic domain. The exact boundaries of a transmembrane domain may vary but most likely by no more than about 5 amino acids at either end of the domain specifically mentioned herein.

"Apo-2 variant" means a biologically active Apo-2 as defined below having at least about 80% amino acid sequence identity with the Apo-2 having the deduced amino acid sequence shown in Fig. 1 (SEQ ID NO:1) for a full-length native sequence human Apo-2 or the sequences identified herein for Apo-2 ECD or death domain. Such Apo-2 variants include, for instance, Apo-2 polypeptides wherein one or more amino acid residues are added, or deleted, at the N- or C-terminus of the sequence of Fig. 1 (SEQ ID NO:1) or the sequences identified herein for Apo-2 ECD or death domain. Ordinarily, an Apo-2 variant will have at least about 80% amino acid sequence identity, more preferably at least about 90% amino acid sequence identity, and even more preferably at least about 95% amino acid sequence identity with the amino acid sequence of Fig. 1 (SEQ ID NO:1) or the sequences identified herein for Apo-2 ECD or death domain.

"Percent (%) amino acid sequence identity" with respect to the Apo-2 sequences identified herein is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the Apo-2 sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as ALIGN™ or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared.

The term "epitope tagged" when used herein refers to a chimeric polypeptide comprising Apo-2 or Apo-2 antibody, or a domain sequence thereof, fused to a "tag polypeptide". The tag

polypeptide has enough residues to provide an epitope against which an antibody can be made, yet is short enough such that it does not interfere with activity of the Apo-2 or Apo-2 antibody. The tag polypeptide preferably also is fairly unique so that the antibody  
 5 does not substantially cross-react with other epitopes. Suitable tag polypeptides generally have at least six amino acid residues and usually between about 8 to about 50 amino acid residues (preferably, between about 10 to about 20 residues).

"Isolated," when used to describe the various  
 10 polypeptides disclosed herein, means polypeptide that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would typically interfere with diagnostic or therapeutic uses for the polypeptide, and may include  
 15 enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In preferred embodiments, the polypeptide will be purified (1) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (2) to homogeneity by SDS-PAGE under non-  
 20 reducing or reducing conditions using Coomassie blue or, preferably, silver stain. Isolated polypeptide includes polypeptide *in situ* within recombinant cells, since at least one component of the Apo-2 natural environment will not be present. Ordinarily, however, isolated polypeptide will be prepared by at  
 25 least one purification step.

An "isolated" Apo-2 nucleic acid molecule is a nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the Apo-2 nucleic acid. An  
 30 isolated Apo-2 nucleic acid molecule is other than in the form or setting in which it is found in nature. Isolated Apo-2 nucleic acid molecules therefore are distinguished from the Apo-2 nucleic acid molecule as it exists in natural cells. However, an isolated Apo-2 nucleic acid molecule includes Apo-2 nucleic acid molecules  
 35 contained in cells that ordinarily express Apo-2 where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence

in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

The term "antibody" is used in the broadest sense and specifically covers anti-Apo-2 monoclonal antibodies (including agonist, antagonist, and blocking or neutralizing antibodies) and anti-Apo-2 antibody compositions with polyepitopic specificity.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally-occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen.

The monoclonal antibodies herein include hybrid and recombinant antibodies produced by splicing a variable (including hypervariable) domain of an anti-Apo-2 antibody with a constant domain, or a light chain with a heavy chain, or a chain from one

species with a chain from another species, or fusions with heterologous proteins, regardless of species of origin or immunoglobulin class or subclass designation, as well as antibody fragments (e.g., Fab, F(ab')<sub>2</sub>, and Fv), so long as they exhibit the desired biological activity. See, e.g. U.S. Pat. No. 4,816,567 and Mage et al., in Monoclonal Antibody Production Techniques and Applications, pp.79-97 (Marcel Dekker, Inc.: New York, 1987).

Thus, the modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler and Milstein, Nature, 256:495 (1975), or may be made by recombinant DNA methods such as described in U.S. Pat. No. 4,816,567. The "monoclonal antibodies" may also be isolated from phage libraries generated using the techniques described in McCafferty et al., Nature, 348:552-554 (1990), for example.

"Single-chain Fv" or "scFv" antibody fragments comprise the V<sub>H</sub> and V<sub>L</sub> domains of antibody, wherein these domains are present in a single polypeptide chain. Generally, the Fv polypeptide further comprises a polypeptide linker between the V<sub>H</sub> and V<sub>L</sub> domains which enables the scFv to form the desired structure for antigen binding. For a review of scFv see, e.g., Pluckthun, The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenberg and Moore eds. Springer-Verlag, New York, pp. 269-315 (1994). The scFv antibody fragments of the present invention include but are not limited to the 16E2, 20E6 and 24C4 antibodies described in detail below. Within the scope of the scFv antibodies of the invention are scFv antibodies comprising V<sub>H</sub> and V<sub>L</sub> domains that include one or more of the CDR regions identified for the 16E2, 20E6 and 24C4 antibodies.

"Humanized" forms of non-human (e.g. murine) antibodies are specific chimeric immunoglobulins, immunoglobulin chains, or fragments thereof (such as Fv, Fab, Fab', F(ab')<sub>2</sub> or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of



the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat, or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, the humanized antibody may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region or domain (Fc), typically that of a human immunoglobulin.

"Biologically active" and "desired biological activity" for the purposes herein means (1) having the ability to modulate apoptosis (either in an agonistic or stimulating manner or in an antagonistic or blocking manner) in at least one type of mammalian cell *in vivo* or *ex vivo*; (2) having the ability to bind Apo-2 ligand; or (3) having the ability to modulate Apo-2 ligand signaling and Apo-2 ligand activity.

The terms "apoptosis" and "apoptotic activity" are used in a broad sense and refer to the orderly or controlled form of cell death in mammals that is typically accompanied by one or more characteristic cell changes, including condensation of cytoplasm, loss of plasma membrane microvilli, segmentation of the nucleus, degradation of chromosomal DNA or loss of mitochondrial function. This activity can be determined and measured, for instance, by cell viability assays, FACS analysis or DNA electrophoresis, all of which are known in the art.

The terms "treating," "treatment," and "therapy" as used herein refer to curative therapy, prophylactic therapy, and preventative therapy.

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer

include but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, blastoma, gastrointestinal cancer, renal cancer, pancreatic cancer, glioblastoma, neuroblastoma, cervical cancer, ovarian cancer, liver cancer, stomach cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer.

The term "mammal" as used herein refers to any mammal classified as a mammal, including humans, cows, horses, dogs and cats. In a preferred embodiment of the invention, the mammal is a human.

## II. Compositions and Methods of the Invention

The present invention provides newly identified and isolated Apo-2 polypeptides and Apo-2 antibodies. In particular, Applicants have identified and isolated various human Apo-2 polypeptides. The properties and characteristics of some of these Apo-2 polypeptides and anti-Apo-2 antibodies are described in further detail in the Examples below. Based upon the properties and characteristics of the Apo-2 polypeptides disclosed herein, it is Applicants' present belief that Apo-2 is a member of the TNFR family.

A description follows as to how Apo-2, as well as Apo-2 chimeric molecules and anti-Apo-2 antibodies, may be prepared.

### A. Preparation of Apo-2

The description below relates primarily to production of Apo-2 by culturing cells transformed or transfected with a vector containing Apo-2 nucleic acid. It is of course, contemplated that alternative methods, which are well known in the art, may be employed to prepare Apo-2.

#### 1. Isolation of DNA Encoding Apo-2

The DNA encoding Apo-2 may be obtained from any cDNA library prepared from tissue believed to possess the Apo-2 mRNA and to express it at a detectable level. Accordingly, human Apo-2 DNA can be conveniently obtained from a cDNA library prepared from

human tissues, such as the bacteriophage libraries of human pancreas and kidney cDNA described in Example 1. The Apo-2-encoding gene may also be obtained from a genomic library or by oligonucleotide synthesis.

5 Libraries can be screened with probes (such as antibodies to the Apo-2 or oligonucleotides of at least about 20-80 bases) designed to identify the gene of interest or the protein encoded by it. Screening the cDNA or genomic library with the selected probe may be conducted using standard procedures, such as  
10 described in Sambrook et al., Molecular Cloning: A Laboratory Manual (New York: Cold Spring Harbor Laboratory Press, 1989). An alternative means to isolate the gene encoding Apo-2 is to use PCR methodology [Sambrook et al., supra; Dieffenbach et al., PCR Primer: A Laboratory Manual (Cold Spring Harbor Laboratory Press,  
15 1995)].

A preferred method of screening employs selected oligonucleotide sequences to screen cDNA libraries from various human tissues. Example 1 below describes techniques for screening a cDNA library. The oligonucleotide sequences selected as probes  
20 should be of sufficient length and sufficiently unambiguous that false positives are minimized. The oligonucleotide is preferably labeled such that it can be detected upon hybridization to DNA in the library being screened. Methods of labeling are well known in the art, and include the use of radiolabels like <sup>32</sup>P-labeled ATP,  
25 biotinylation or enzyme labeling. Hybridization conditions, including moderate stringency and high stringency, are provided in Sambrook et al., supra.

Nucleic acid having all the protein coding sequence may be obtained by screening selected cDNA or genomic libraries using  
30 the deduced amino acid sequence disclosed herein for the first time, and, if necessary, using conventional primer extension procedures as described in Sambrook et al., supra, to detect precursors and processing intermediates of mRNA that may not have been reverse-transcribed into cDNA.

35 Apo-2 variants can be prepared by introducing appropriate nucleotide changes into the Apo-2 DNA, or by synthesis of the desired Apo-2 polypeptide. Those skilled in the art will appreciate that amino acid changes may alter post-translational processes of the Apo-2, such as changing the number or position of

glycosylation sites or altering the membrane anchoring characteristics.

Variations in the native full-length sequence Apo-2 or in various domains of the Apo-2 described herein, can be made, for example, using any of the techniques and guidelines for conservative and non-conservative mutations set forth, for instance, in U.S. Pat. No. 5,364,934. Variations may be a substitution, deletion or insertion of one or more codons encoding the Apo-2 that results in a change in the amino acid sequence of the Apo-2 as compared with the native sequence Apo-2. Optionally the variation is by substitution of at least one amino acid with any other amino acid in one or more of the domains of the Apo-2 molecule. The variations can be made using methods known in the art such as oligonucleotide-mediated (site-directed) mutagenesis, alanine scanning, and PCR mutagenesis. Site-directed mutagenesis [Carter et al., Nucl. Acids Res., 13:4331 (1986); Zoller et al., Nucl. Acids Res., 10:6487 (1987)], cassette mutagenesis [Wells et al., Gene, 34:315 (1985)], restriction selection mutagenesis [Wells et al., Philos. Trans. R. Soc. London SerA, 317:415 (1986)] or other known techniques can be performed on the cloned DNA to produce the Apo-2 variant DNA.

Scanning amino acid analysis can also be employed to identify one or more amino acids along a contiguous sequence which are involved in the interaction with a particular ligand or receptor. Among the preferred scanning amino acids are relatively small, neutral amino acids. Such amino acids include alanine, glycine, serine, and cysteine. Alanine is the preferred scanning amino acid among this group because it eliminates the side-chain beyond the beta-carbon and is less likely to alter the main-chain conformation of the variant. Alanine is also preferred because it is the most common amino acid. Further, it is frequently found in both buried and exposed positions [Creighton, The Proteins, (W.H. Freeman & Co., N.Y.); Chothia, J. Mol. Biol., 150:1 (1976)]. If alanine substitution does not yield adequate amounts of variant, an isoteric amino acid can be used.

Once selected Apo-2 variants are produced, they can be contacted with, for instance, Apo-2L, and the interaction, if any, can be determined. The interaction between the Apo-2 variant and Apo-2L can be measured by an *in vitro* assay, such as described in

the Examples below. While any number of analytical measurements can be used to compare activities and properties between a native sequence Apo-2 and an Apo-2 variant, a convenient one for binding is the dissociation constant  $K_d$  of the complex formed between the Apo-2 variant and Apo-2L as compared to the  $K_d$  for the native sequence Apo-2. Generally, a  $\geq 3$ -fold increase or decrease in  $K_d$  per substituted residue indicates that the substituted residue(s) is active in the interaction of the native sequence Apo-2 with the Apo-2L.

Optionally, representative sites in the Apo-2 sequence suitable for mutagenesis would include sites within the extracellular domain, and particularly, within one or both of the cysteine-rich domains. Such variations can be accomplished using the methods described above.

## 2. Insertion of Nucleic Acid into A Replicable Vector

The nucleic acid (e.g., cDNA or genomic DNA) encoding Apo-2 may be inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. Various vectors are publicly available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence, each of which is described below.

### (i) Signal Sequence Component

The Apo-2 may be produced recombinantly not only directly, but also as a fusion polypeptide with a heterologous polypeptide, which may be a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. In general, the signal sequence may be a component of the vector, or it may be a part of the Apo-2 DNA that is inserted into the vector. The heterologous signal sequence selected preferably is one that is recognized and processed (i.e., cleaved by a signal peptidase) by the host cell. The signal sequence may be a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion the signal sequence may be, e.g., the yeast invertase leader, alpha factor leader (including *Saccharomyces* and *Kluyveromyces*  $\alpha$ -factor leaders, the latter described in U.S. Pat. No. 5,010,182), or acid

phosphatase leader, the *C. albicans* glucoamylase leader (EP 362,179 published 4 April 1990), or the signal described in WO 90/13646 published 15 November 1990. In mammalian cell expression the native Apo-2 presequence that normally directs insertion of Apo-2 in the cell membrane of human cells *in vivo* is satisfactory, although other mammalian signal sequences may be used to direct secretion of the protein, such as signal sequences from secreted polypeptides of the same or related species, as well as viral secretory leaders, for example, the herpes simplex glycoprotein D signal.

The DNA for such precursor region is preferably ligated in reading frame to DNA encoding Apo-2.

#### (ii) Origin of Replication Component

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2 $\mu$  plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used because it contains the early promoter).

Most expression vectors are "shuttle" vectors, i.e., they are capable of replication in at least one class of organisms but can be transfected into another organism for expression. For example, a vector is cloned in *E. coli* and then the same vector is transfected into yeast or mammalian cells for expression even though it is not capable of replicating independently of the host cell chromosome.

DNA may also be amplified by insertion into the host genome. This is readily accomplished using *Bacillus* species as hosts, for example, by including in the vector a DNA sequence that is complementary to a sequence found in *Bacillus* genomic DNA. Transfection of *Bacillus* with this vector results in homologous

recombination with the genome and insertion of Apo-2 DNA. However, the recovery of genomic DNA encoding Apo-2 is more complex than that of an exogenously replicated vector because restriction enzyme digestion is required to excise the Apo-2 DNA.

5

(iii) Selection Gene Component

Expression and cloning vectors typically contain a selection gene, also termed a selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g., the gene encoding D-alanine racemase for *Bacilli*.

One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene produce a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin [Southern et al., *J. Molec. Appl. Genet.*, 1:327 (1982)], mycophenolic acid [Mulligan et al., *Science*, 209:1422 (1980)] or hygromycin [Sugden et al., *Mol. Cell. Biol.*, 5:410-413 (1985)]. The three examples given above employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug G418 or neomycin (geneticin), xgpt (mycophenolic acid), or hygromycin, respectively.

Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the Apo-2 nucleic acid, such as DHFR or thymidine kinase. The mammalian cell transformants are placed under selection pressure that only the transformants are uniquely adapted to survive by virtue of having taken up the marker. Selection pressure is imposed by culturing the transformants under conditions in which the concentration of selection agent in the medium is successively changed, thereby leading to amplification of both the selection gene and the DNA that encodes Apo-2. Amplification is the process by which genes in greater demand for the production of a protein critical for growth are reiterated in

tandem within the chromosomes of successive generations of recombinant cells. Increased quantities of Apo-2 are synthesized from the amplified DNA. Other examples of amplifiable genes include metallothionein-I and -II, adenosine deaminase, and  
5 ornithine decarboxylase.

Cells transformed with the DHFR selection gene may first be identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed  
10 is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub et al., Proc. Natl. Acad. Sci. USA, 77:4216 (1980). The transformed cells are then exposed to increased levels of methotrexate. This leads to the synthesis of multiple copies of the DHFR gene, and,  
15 concomitantly, multiple copies of other DNA comprising the expression vectors, such as the DNA encoding Apo-2. This amplification technique can be used with any otherwise suitable host, e.g., ATCC No. CCL61 CHO-K1, notwithstanding the presence of endogenous DHFR if, for example, a mutant DHFR gene that is highly  
20 resistant to Mtx is employed (EP 117,060).

Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding Apo-2, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3'-phosphotransferase  
25 (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, e.g., kanamycin, neomycin, or G418. See U.S. Patent No. 4,965,199.

A suitable selection gene for use in yeast is the *trp1* gene present in the yeast plasmid YRp7 [Stinchcomb et al., Nature, 282:39 (1979); Kingsman et al., Gene, 7:141 (1979); Tschemper et al., Gene, 10:157 (1980)]. The *trp1* gene provides a selection  
30 marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1 [Jones, Genetics, 85:12 (1977)]. The presence of the *trp1* lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, *Leu2*-deficient yeast strains (ATCC 20,622 or 38,626) are  
35 complemented by known plasmids bearing the *Leu2* gene.



In addition, vectors derived from the 1.6  $\mu$ m circular plasmid pKD1 can be used for transformation of *Kluyveromyces* yeasts [Bianchi et al., Curr. Genet., 12:185 (1987)]. More recently, an expression system for large-scale production of recombinant calf chymosin was reported for *K. lactis* [Van den Berg, Bio/Technology, 8:135 (1990)]. Stable multi-copy expression vectors for secretion of mature recombinant human serum albumin by industrial strains of *Kluyveromyces* have also been disclosed [Fleer et al., Bio/Technology, 9:968-975 (1991)].

10 (iv) Promoter Component

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the Apo-2 nucleic acid sequence. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of particular nucleic acid sequence, such as the Apo-2 nucleic acid sequence, to which they are operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, e.g., the presence or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These promoters are operably linked to Apo-2 encoding DNA by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector. Both the native Apo-2 promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of the Apo-2 DNA.

Promoters suitable for use with prokaryotic hosts include the  $\beta$ -lactamase and lactose promoter systems [Chang et al., Nature, 275:615 (1978); Goeddel et al., Nature, 281:544 (1979)], alkaline phosphatase, a tryptophan (trp) promoter system [Goeddel, Nucleic Acids Res., 8:4057 (1980); EP 36,776], and hybrid promoters such as the tac promoter [deBoer et al., Proc. Natl. Acad. Sci. USA, 80:21-25 (1983)]. However, other known bacterial promoters are suitable. Their nucleotide sequences have been published,

thereby enabling a skilled worker operably to ligate them to DNA encoding Apo-2 [Siebenlist et al., Cell, 20:269 (1980)] using linkers or adaptors to supply any required restriction sites. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding Apo-2.

Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CXCAAT region where X may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into eukaryotic expression vectors.

Examples of suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase [Hitzeman et al., J. Biol. Chem., 255:2073 (1980)] or other glycolytic enzymes [Hess et al., J. Adv. Enzyme Reg., 7:149 (1968); Holland, Biochemistry, 17:4900 (1978)], such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytocrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in EP 73,657. Yeast enhancers also are advantageously used with yeast promoters.

Apo-2 transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published 5 July 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40

(SV40), from heterologous mammalian promoters, e.g., the actin promoter or an immunoglobulin promoter, from heat-shock promoters, and from the promoter normally associated with the Apo-2 sequence, provided such promoters are compatible with the host cell systems.

5           The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication [Fiers et al., Nature, 273:113 (1978); Mulligan and Berg, Science, 209:1422-1427 (1980); Pavlakis et al., Proc. Natl. Acad. Sci. USA, 78:7398-7402 (1981)]. The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment [Greenaway et al., Gene, 18:355-360 (1982)]. A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. Patent No. 4,419,446. A  
10           modification of this system is described in U.S. Patent No. 4,601,978 [See also Gray et al., Nature, 295:503-508 (1982) on expressing cDNA encoding immune interferon in monkey cells; Reyes et al., Nature, 297:598-601 (1982) on expression of human  $\beta$ -interferon cDNA in mouse cells under the control of a thymidine  
20           kinase promoter from herpes simplex virus; Canaani and Berg, Proc. Natl. Acad. Sci. USA 79:5166-5170 (1982) on expression of the human interferon gene in cultured mouse and rabbit cells; and Gorman et al., Proc. Natl. Acad. Sci. USA, 79:6777-6781 (1982) on expression of bacterial CAT sequences in CV-1 monkey kidney cells, chicken  
25           embryo fibroblasts, Chinese hamster ovary cells, HeLa cells, and mouse NIH-3T3 cells using the Rous sarcoma virus long terminal repeat as a promoter].

(v) Enhancer Element Component

30           Transcription of a DNA encoding the Apo-2 of this invention by higher eukaryotes may be increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp, that act on a promoter to increase its transcription. Enhancers are relatively orientation and position independent, having been found 5' [Laimins et al., Proc. Natl. Acad. Sci. USA, 78:993 (1981)] and 3' [Lusky et al., Mol. Cell Bio., 3:1108 (1983)] to the transcription unit, within an intron [Banerji et al., Cell, 33:729 (1983)], as well as within the coding sequence itself [Osborne et al., Mol. Cell Bio., 4:1293 (1984)]. Many enhancer sequences are now known from  
35

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mammalian genes (globin, elastase, albumin,  $\alpha$ -fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the  
5 cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, Nature, 297:17-18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to the Apo-2 coding  
10 sequence, but is preferably located at a site 5' from the promoter.

(vi) Transcription Termination Component

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for  
15 the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA  
20 encoding Apo-2.

(vii) Construction and Analysis of Vectors

Construction of suitable vectors containing one or more of the above-listed components employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved,  
25 tailored, and re-ligated in the form desired to generate the plasmids required.

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures can be used to transform *E. coli* K12 strain 294 (ATCC 31,446) and successful transformants selected  
30 by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced by the method of Messing et al., Nucleic Acids Res., 9:309 (1981) or by the method of Maxim et al., Methods in Enzymology, 65:499 (1980).

35 (viii) Transient Expression Vectors

Expression vectors that provide for the transient expression in mammalian cells of DNA encoding Apo-2 may be employed. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host

cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector [Sambrook et al., *supra*]. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of such polypeptides for desired biological or physiological properties. Thus, transient expression systems are particularly useful in the invention for purposes of identifying Apo-2 variants.

(ix) Suitable Exemplary Vertebrate Cell Vectors

Other methods, vectors, and host cells suitable for adaptation to the synthesis of Apo-2 in recombinant vertebrate cell culture are described in Gething et al., Nature, 293:620-625 (1981); Mantei et al., Nature, 281:40-46 (1979); EP 117,060; and EP 117,058.

3. Selection and Transformation of Host Cells

Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include but are not limited to eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as *Escherichia*, e.g., *E. coli*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Proteus*, *Salmonella*, e.g., *Salmonella typhimurium*, *Serratia*, e.g., *Serratia marcescans*, and *Shigella*, as well as *Bacilli* such as *B. subtilis* and *B. licheniformis* (e.g., *B. licheniformis* 41P disclosed in DD 266,710 published 12 April 1989), *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*. Preferably, the host cell should secrete minimal amounts of proteolytic enzymes.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for Apo-2-encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein.

Suitable host cells for the expression of glycosylated Apo-2 are derived from multicellular organisms. Such host cells are capable of complex processing and glycosylation activities. In principle, any higher eukaryotic cell culture is workable, whether

from vertebrate or invertebrate culture. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified [See, e.g., Luckow et al., Bio/Technology, 6:47-55 (1988); Miller et al., in Genetic Engineering, Setlow et al., eds., Vol. 8 (Plenum Publishing, 1986), pp. 277-279; and Maeda et al., Nature, 315:592-594 (1985)]. A variety of viral strains for transfection are publicly available, e.g., the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV.

Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can be utilized as hosts. Typically, plant cells are transfected by incubation with certain strains of the bacterium *Agrobacterium tumefaciens*. During incubation of the plant cell culture with *A. tumefaciens*, the DNA encoding the Apo-2 can be transferred to the plant cell host such that it is transfected, and will, under appropriate conditions, express the Apo-2-encoding DNA. In addition, regulatory and signal sequences compatible with plant cells are available, such as the nopaline synthase promoter and polyadenylation signal sequences [Depicker et al., J. Mol. Appl. Gen., 1:561 (1982)]. In addition, DNA segments isolated from the upstream region of the T-DNA 780 gene are capable of activating or increasing transcription levels of plant-expressible genes in recombinant DNA-containing plant tissue [EP 321,196 published 21 June 1989].

Propagation of vertebrate cells in culture (tissue culture) is also well known in the art [See, e.g., Tissue Culture, Academic Press, Kruse and Patterson, editors (1973)]. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., J. Gen. Virol., 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, Proc. Natl. Acad. Sci. USA, 77:4216 (1980)); mouse sertoli cells (TM4, Mather, Biol. Reprod., 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical

carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells  
5 (Mather et al., Annals N.Y. Acad. Sci., 383:44-68 (1982)); MRC 5 cells; and FS4 cells.

Host cells are transfected and preferably transformed with the above-described expression or cloning vectors for Apo-2 production and cultured in conventional nutrient media modified as  
10 appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the  
15 ordinarily skilled artisan, for example, CaPO<sub>4</sub> and electroporation. Successful transfection is generally recognized when any indication of the operation of this vector occurs within the host cell.

Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element  
20 or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in Sambrook et al., supra, or electroporation is generally used for prokaryotes or other cells that contain  
25 substantial cell-wall barriers. Infection with *Agrobacterium tumefaciens* is used for transformation of certain plant cells, as described by Shaw et al., Gene, 23:315 (1983) and WO 89/05859 published 29 June 1989. In addition, plants may be transfected using ultrasound treatment as described in WO 91/00358 published 10  
30 January 1991.

For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb, Virology, 52:456-457 (1978) is preferred. General aspects of mammalian cell host system transformations have been described in U.S. Pat. No.  
35 4,399,216. Transformations into yeast are typically carried out according to the method of Van Solingen et al., J. Bact., 130:946 (1977) and Hsiao et al., Proc. Natl. Acad. Sci. (USA), 76:3829 (1979). However, other methods for introducing DNA into cells, such as by nuclear microinjection, electroporation, bacterial

protoplast fusion with intact cells, or polycations, e.g., polybrene, polyornithine, may also be used. For various techniques for transforming mammalian cells, see Keown et al., Methods in Enzymology, 185:527-537 (1990) and Mansour et al., Nature, 336:348-352 (1988).

#### 4. Culturing the Host Cells

Prokaryotic cells used to produce Apo-2 may be cultured in suitable media as described generally in Sambrook et al., supra.

The mammalian host cells used to produce Apo-2 may be cultured in a variety of media. Examples of commercially available media include Ham's F10 (Sigma), Minimal Essential Medium ("MEM", Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ("DMEM", Sigma). Any such media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

In general, principles, protocols, and practical techniques for maximizing the productivity of mammalian cell cultures can be found in Mammalian Cell Biotechnology: a Practical Approach, M. Butler, ed. (IRL Press, 1991).

The host cells referred to in this disclosure encompass cells in culture as well as cells that are within a host animal.

#### 5. Detecting Gene Amplification/Expression

Gene amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA [Thomas, Proc. Natl. Acad. Sci. USA, 77:5201-5205 (1980)], dot blotting (DNA analysis), or *in situ* hybridization, using an appropriately labeled probe, based on the sequences provided herein. Various labels may be employed, most commonly radioisotopes, and particularly <sup>32</sup>P.



However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionucleotides, fluorescers or enzymes. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of cells or tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. With immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and fixation, followed by reaction with labeled antibodies specific for the gene product coupled, where the labels are usually visually detectable, such as enzymatic labels, fluorescent labels, or luminescent labels.

Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared in any mammal. Conveniently, the antibodies may be prepared against a native sequence Apo-2 polypeptide or against a synthetic peptide based on the DNA sequences provided herein or against exogenous sequence fused to Apo-2 DNA and encoding a specific antibody epitope.

#### 6. Purification of Apo-2 Polypeptide

Forms of Apo-2 may be recovered from culture medium or from host cell lysates. If the Apo-2 is membrane-bound, it can be released from the membrane using a suitable detergent solution (e.g. Triton-X 100) or its extracellular domain may be released by enzymatic cleavage.

When Apo-2 is produced in a recombinant cell other than one of human origin, the Apo-2 is free of proteins or polypeptides of human origin. However, it may be desired to purify Apo-2 from recombinant cell proteins or polypeptides to obtain preparations that are substantially homogeneous as to Apo-2. As a first step, the culture medium or lysate may be centrifuged to remove

particulate cell debris. Apo-2 thereafter is purified from contaminant soluble proteins and polypeptides, with the following procedures being exemplary of suitable purification procedures: by fractionation on an ion-exchange column; ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; and protein A Sepharose columns to remove contaminants such as IgG.

Apo-2 variants in which residues have been deleted, inserted, or substituted can be recovered in the same fashion as native sequence Apo-2, taking account of changes in properties occasioned by the variation. For example, preparation of an Apo-2 fusion with another protein or polypeptide, e.g., a bacterial or viral antigen, immunoglobulin sequence, or receptor sequence, may facilitate purification; an immunoaffinity column containing antibody to the sequence can be used to adsorb the fusion polypeptide. Other types of affinity matrices also can be used.

A protease inhibitor such as phenyl methyl sulfonyl fluoride (PMSF) also may be useful to inhibit proteolytic degradation during purification, and antibiotics may be included to prevent the growth of adventitious contaminants. One skilled in the art will appreciate that purification methods suitable for native sequence Apo-2 may require modification to account for changes in the character of Apo-2 or its variants upon expression in recombinant cell culture.

#### 7. Covalent Modifications of Apo-2 Polypeptides

Covalent modifications of Apo-2 are included within the scope of this invention. One type of covalent modification of the Apo-2 is introduced into the molecule by reacting targeted amino acid residues of the Apo-2 with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues of the Apo-2.

Derivatization with bifunctional agents is useful for crosslinking Apo-2 to a water-insoluble support matrix or surface for use in the method for purifying anti-Apo-2 antibodies, and vice-versa. Derivatization with one or more bifunctional agents will also be useful for crosslinking Apo-2 molecules to generate Apo-2 dimers. Such dimers may increase binding avidity and extend

half-life of the molecule *in vivo*. Commonly used crosslinking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including  
5 disuccinimidyl esters such as 3,3'-dithiobis(succinimidyl-propionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)-dithio]propioimide yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light.  
10 Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Patent Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

15 Other modifications include deamidation of glutamyl and asparagyl residues to the corresponding glutamyl and aspartyl residues, respectively, hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the  $\alpha$ -amino groups of lysine, arginine, and  
20 histidine side chains [T.E. Creighton, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)], acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group. The modified forms of the residues fall within the scope of the present invention.

25 Another type of covalent modification of the Apo-2 polypeptide included within the scope of this invention comprises altering the native glycosylation pattern of the polypeptide. "Altering the native glycosylation pattern" is intended for purposes herein to mean deleting one or more carbohydrate moieties  
30 found in native sequence Apo-2, and/or adding one or more glycosylation sites that are not present in the native sequence Apo-2.

Glycosylation of polypeptides is typically either N-linked or O-linked. N-linked refers to the attachment of the  
35 carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either

of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-acetylglactosamine, galactose, or xylose to a hydroxylamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Addition of glycosylation sites to the Apo-2 polypeptide may be accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the native sequence Apo-2 (for O-linked glycosylation sites). The Apo-2 amino acid sequence may optionally be altered through changes at the DNA level, particularly by mutating the DNA encoding the Apo-2 polypeptide at preselected bases such that codons are generated that will translate into the desired amino acids. The DNA mutation(s) may be made using methods described above and in U.S. Pat. No. 5,364,934, supra.

Another means of increasing the number of carbohydrate moieties on the Apo-2 polypeptide is by chemical or enzymatic coupling of glycosides to the polypeptide. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. These methods are described in WO 87/05330 published 11 September 1987, and in Aplin and Wriston, CRC Crit. Rev. Biochem., pp. 259-306 (1981).

Removal of carbohydrate moieties present on the Apo-2 polypeptide may be accomplished chemically or enzymatically or by mutational substitution of codons encoding for amino acid residues that serve as targets for glycosylation. For instance, chemical deglycosylation by exposing the polypeptide to the compound trifluoromethanesulfonic acid, or an equivalent compound can result in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylglactosamine), while leaving the polypeptide intact. Chemical deglycosylation is described by Hakimuddin, et al., Arch. Biochem. Biophys., 259:52 (1987) and by Edge et al., Anal. Biochem., 118:131 (1981). Enzymatic cleavage of

carbohydrate moieties on polypeptides can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura et al., Meth. Enzymol., 138:350 (1987).

Glycosylation at potential glycosylation sites may be prevented by the use of the compound tunicamycin as described by Duksin et al., J. Biol. Chem., 257:3105 (1982). Tunicamycin blocks the formation of protein-N-glycoside linkages.

Another type of covalent modification of Apo-2 comprises linking the Apo-2 polypeptide to one of a variety of nonproteinaceous polymers, e.g., polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

#### 8. Apo-2 Chimeras

The present invention also provides chimeric molecules comprising Apo-2 fused to another, heterologous polypeptide or amino acid sequence.

In one embodiment, the chimeric molecule comprises a fusion of the Apo-2 with a tag polypeptide which provides an epitope to which an anti-tag antibody can selectively bind. The epitope tag is generally placed at the amino- or carboxyl- terminus of the Apo-2. The presence of such epitope-tagged forms of the Apo-2 can be detected using an antibody against the tag polypeptide. Also, provision of the epitope tag enables the Apo-2 to be readily purified by affinity purification using an anti-tag antibody or another type of affinity matrix that binds to the epitope tag.

Various tag polypeptides and their respective antibodies are well known in the art. Examples include the flu HA tag polypeptide and its antibody 12CA5 [Field et al., Mol. Cell. Biol., 8:2159-2165 (1988)]; the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto [Evan et al., Molecular and Cellular Biology, 5:3610-3616 (1985)]; and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody [Paborsky et al., Protein Engineering, 3(6):547-553 (1990)]. Other tag polypeptides include the Flag-peptide [Hopp et al., BioTechnology, 6:1204-1210 (1988)]; the KT3 epitope peptide [Martin et al., Science, 255:192-194 (1992)]; an  $\alpha$ -tubulin epitope peptide [Skinner et al., J. Biol. Chem., 266:15163-15166 (1991)]; and the T7 gene 10 protein peptide

tag [Lutz-Freyermuth et al., Proc. Natl. Acad. Sci. USA, 87:6393-6397 (1990)]. Once the tag polypeptide has been selected, an antibody thereto can be generated using the techniques disclosed herein.

5           Generally, epitope-tagged Apo-2 may be constructed and produced according to the methods described above. Epitope-tagged Apo-2 is also described in the Examples below. Apo-2-tag polypeptide fusions are preferably constructed by fusing the cDNA sequence encoding the Apo-2 portion in-frame to the tag polypeptide  
10 DNA sequence and expressing the resultant DNA fusion construct in appropriate host cells. Ordinarily, when preparing the Apo-2-tag polypeptide chimeras of the present invention, nucleic acid encoding the Apo-2 will be fused at its 3' end to nucleic acid encoding the N-terminus of the tag polypeptide, however 5' fusions  
15 are also possible. For example, a polyhistidine sequence of about 5 to about 10 histidine residues may be fused at the N- terminus or the C- terminus and used as a purification handle in affinity chromatography.

          Epitope-tagged Apo-2 can be purified by affinity  
20 chromatography using the anti-tag antibody. The matrix to which the affinity antibody is attached may include, for instance, agarose, controlled pore glass or poly(styrenedivinyl)benzene. The epitope-tagged Apo-2 can then be eluted from the affinity column using techniques known in the art.

25           In another embodiment, the chimeric molecule comprises an Apo-2 polypeptide fused to an immunoglobulin sequence. The chimeric molecule may also comprise a particular domain sequence of Apo-2, such as an extracellular domain sequence of Apo-2 fused to an immunoglobulin sequence. This includes chimeras in monomeric,  
30 homo- or heteromultimeric, and particularly homo- or heterodimeric, or -tetrameric forms; optionally, the chimeras may be in dimeric forms or homodimeric heavy chain forms. Generally, these assembled immunoglobulins will have known unit structures as represented by the following diagrams.

X or A

\ \_\_\_\_\_ C<sub>H</sub> or C<sub>L</sub>

5

X or A

\ \_\_\_\_\_ Y \_\_\_\_\_ C<sub>H</sub> or C<sub>L</sub>

10

A

A \ \_\_\_\_\_ C<sub>L</sub>

\ \_\_\_\_\_ C<sub>H</sub>

15

A

V<sub>H</sub> \ \_\_\_\_\_ C<sub>L</sub>

\ \_\_\_\_\_ C<sub>H</sub>

20

V<sub>L</sub>

A \ \_\_\_\_\_ C<sub>L</sub>

\ \_\_\_\_\_ C<sub>H</sub>

25

X

A \ \_\_\_\_\_ C<sub>L</sub>

\ \_\_\_\_\_ C<sub>H</sub>

30

A

X \ \_\_\_\_\_ C<sub>L</sub>

\ \_\_\_\_\_ C<sub>H</sub>

35 A basic four chain structural unit is the form in which  
IgG, IgD, and IgE exist. A four chain unit is repeated in the  
higher molecular weight immunoglobulins; IgM generally exists as a  
pentamer of basic four-chain units held together by disulfide  
bonds. IgA globulin, and occasionally IgG globulin, may also exist

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in a multimeric form in serum. In the case of multimers, each four chain unit may be the same or different.

The following diagrams depict some exemplary monomer, homo- and heterodimer and homo- and heteromultimer structures.

- 5 These diagrams are merely illustrative, and the chains of the multimers are believed to be disulfide bonded in the same fashion as native immunoglobulins.



monomer:

A \_\_\_\_\_ C<sub>L</sub> or C<sub>H</sub>

5 homodimer:

A  
 \ \_\_\_\_\_ C<sub>L</sub> or C<sub>H</sub>  
 \_\_\_\_\_ C<sub>L</sub> or C<sub>H</sub>  
 /  
 A

10

heterodimer:

A  
 \ \_\_\_\_\_ C<sub>L</sub> or C<sub>H</sub>  
 \_\_\_\_\_ C<sub>L</sub> or C<sub>H</sub>  
 /  
 X

15

homotetramer:

A  
 A \ \_\_\_\_\_ C<sub>L</sub>  
 \ \_\_\_\_\_ C<sub>L</sub> or C<sub>H</sub>  
 \_\_\_\_\_ C<sub>L</sub> or C<sub>H</sub>  
 / \_\_\_\_\_ C<sub>L</sub>  
 A /  
 A

20

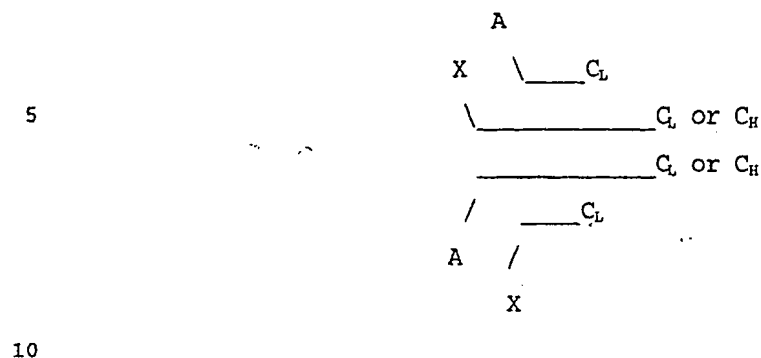
25

heterotetramer:

A  
 A \ \_\_\_\_\_ C<sub>L</sub>  
 \ \_\_\_\_\_ C<sub>L</sub> or C<sub>H</sub>  
 \_\_\_\_\_ C<sub>L</sub> or C<sub>H</sub>  
 / \_\_\_\_\_ C<sub>L</sub>  
 X /  
 X

30

and



15 In the foregoing diagrams, "A" means an Apo-2 sequence or an Apo-2 sequence fused to a heterologous sequence; X is an additional agent, which may be the same as A or different, a portion of an immunoglobulin superfamily member such as a variable region or a variable region-like domain, including a native or chimeric immunoglobulin variable region, a toxin such a pseudomonas exotoxin or ricin, or a sequence functionally binding to another protein, such as other cytokines (i.e., IL-1, interferon- $\gamma$ ) or cell surface molecules (i.e., NGFR, CD40, OX40, Fas antigen, T2 proteins of Shope and myxoma poxviruses), or a polypeptide therapeutic agent not otherwise normally associated with a constant domain; Y is a linker or another receptor sequence; and  $V_L$ ,  $V_H$ ,  $C_L$  and  $C_H$  represent light or heavy chain variable or constant domains of an immunoglobulin. Structures comprising at least one CRD of an Apo-2 sequence as "A" and another cell-surface protein having a repetitive pattern of CRDs (such as TNFR) as "X" are specifically included.

20 It will be understood that the above diagrams are merely exemplary of the possible structures of the chimeras of the present invention, and do not encompass all possibilities. For example, there might desirably be several different "A"s, "X"s, or "Y"s in any of these constructs. Also, the heavy or light chain constant domains may be originated from the same or different immunoglobulins. All possible permutations of the illustrated and similar structures are all within the scope of the invention herein.

25 In general, the chimeric molecules can be constructed in a fashion similar to chimeric antibodies in which a variable domain

from an antibody of one species is substituted for the variable domain of another species. See, for example, EP 0 125 023; EP 173,494; Munro, Nature, 312:597 (13 December 1984); Neuberger et al., Nature, 312:604-608 (13 December 1984); Sharon et al., Nature, 309:364-367 (24 May 1984); Morrison et al., Proc. Nat'l. Acad. Sci. USA, 81:6851-6855 (1984); Morrison et al., Science, 229:1202-1207 (1985); Boulianne et al., Nature, 312:643-646 (13 December 1984); Capon et al., Nature, 337:525-531 (1989); Traunecker et al., Nature, 339:68-70 (1989).

Alternatively, the chimeric molecules may be constructed as follows. The DNA including a region encoding the desired sequence, such as an Apo-2 and/or TNFR sequence, is cleaved by a restriction enzyme at or proximal to the 3' end of the DNA encoding the immunoglobulin-like domain(s) and at a point at or near the DNA encoding the N-terminal end of the Apo-2 or TNFR polypeptide (where use of a different leader is contemplated) or at or proximal to the N-terminal coding region for TNFR (where the native signal is employed). This DNA fragment then is readily inserted proximal to DNA encoding an immunoglobulin light or heavy chain constant region and, if necessary, the resulting construct tailored by deletional mutagenesis. Preferably, the Ig is a human immunoglobulin when the chimeric molecule is intended for *in vivo* therapy for humans. DNA encoding immunoglobulin light or heavy chain constant regions is known or readily available from cDNA libraries or is synthesized. See for example, Adams et al., Biochemistry, 19:2711-2719 (1980); Gough et al., Biochemistry, 19:2702-2710 (1980); Dolby et al., Proc. Natl. Acad. Sci. USA, 77:6027-6031 (1980); Rice et al., Proc. Natl. Acad. Sci., 79:7862-7865 (1982); Falkner et al., Nature, 298:286-288 (1982); and Morrison et al., Ann. Rev. Immunol., 2:239-256 (1984).

Further details of how to prepare such fusions are found in publications concerning the preparation of immunoadhesins. Immunoadhesins in general, and CD4-Ig fusion molecules specifically are disclosed in WO 89/02922, published 6 April 1989. Molecules comprising the extracellular portion of CD4, the receptor for human immunodeficiency virus (HIV), linked to IgG heavy chain constant region are known in the art and have been found to have a markedly longer half-life and lower clearance than the soluble extracellular portion of CD4 [Capon et al., *supra*; Byrn et al., Nature, 344:667

(1990)]. The construction of specific chimeric TNFR-IgG molecules is also described in Ashkenazi et al. Proc. Natl. Acad. Sci., 88:10535-10539 (1991); Lesslauer et al. [J. Cell. Biochem. Supplement 15F, 1991, p. 115 (P 432)]; and Peppel and Beutler, J. Cell. Biochem. Supplement 15F, 1991, p. 118 (P 439)].

#### B. Therapeutic and Non-therapeutic Uses for Apo-2

Apo-2, as disclosed in the present specification, can be employed therapeutically to induce apoptosis in mammalian cells. This therapy can be accomplished for instance, using *in vivo* or *ex vivo* gene therapy techniques and includes the use of the death domain sequences disclosed herein. The Apo-2 chimeric molecules (including the chimeric molecules containing an extracellular domain sequence of Apo-2) comprising immunoglobulin sequences can also be employed therapeutically to inhibit apoptosis or NF-KB induction by Apo-2L or by another ligand that Apo-2 binds to.

The Apo-2 of the invention also has utility in non-therapeutic applications. Nucleic acid sequences encoding the Apo-2 may be used as a diagnostic for tissue-specific typing. For example, procedures like *in situ* hybridization, Northern and Southern blotting, and PCR analysis may be used to determine whether DNA and/or RNA encoding Apo-2 is present in the cell type(s) being evaluated. Apo-2 nucleic acid will also be useful for the preparation of Apo-2 by the recombinant techniques described herein.

The isolated Apo-2 may be used in quantitative diagnostic assays as a control against which samples containing unknown quantities of Apo-2 may be prepared. Apo-2 preparations are also useful in generating antibodies, as standards in assays for Apo-2 (e.g., by labeling Apo-2 for use as a standard in a radioimmunoassay, radioreceptor assay, or enzyme-linked immunoassay), in affinity purification techniques, and in competitive-type receptor binding assays when labeled with, for instance, radioiodine, enzymes, or fluorophores.

Modified forms of the Apo-2, such as the Apo-2-IgG chimeric molecules (immunoadhesins) described above, can be used as immunogens in producing anti-Apo-2 antibodies.

Nucleic acids which encode Apo-2 or its modified forms can also be used to generate either transgenic animals or "knock

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out" animals which, in turn, are useful in the development and screening of therapeutically useful reagents. A transgenic animal (e.g., a mouse or rat) is an animal having cells that contain a transgene, which transgene was introduced into the animal or an ancestor of the animal at a prenatal, e.g., an embryonic stage. A transgene is a DNA which is integrated into the genome of a cell from which a transgenic animal develops. In one embodiment, cDNA encoding Apo-2 or an appropriate sequence thereof (such as Apo-2-IgG) can be used to clone genomic DNA encoding Apo-2 in accordance with established techniques and the genomic sequences used to generate transgenic animals that contain cells which express DNA encoding Apo-2. Methods for generating transgenic animals, particularly animals such as mice or rats, have become conventional in the art and are described, for example, in U.S. Patent Nos. 4,736,866 and 4,870,009. Typically, particular cells would be targeted for Apo-2 transgene incorporation with tissue-specific enhancers. Transgenic animals that include a copy of a transgene encoding Apo-2 introduced into the germ line of the animal at an embryonic stage can be used to examine the effect of increased expression of DNA encoding Apo-2. Such animals can be used as tester animals for reagents thought to confer protection from, for example, pathological conditions associated with excessive apoptosis. In accordance with this facet of the invention, an animal is treated with the reagent and a reduced incidence of the pathological condition, compared to untreated animals bearing the transgene, would indicate a potential therapeutic intervention for the pathological condition. In another embodiment, transgenic animals that carry a soluble form of Apo-2 such as an Apo-2 ECD or an immunoglobulin chimera of such form could be constructed to test the effect of chronic neutralization of Apo-2L, a ligand of Apo-2.

Alternatively, non-human homologues of Apo-2 can be used to construct an Apo-2 "knock out" animal which has a defective or altered gene encoding Apo-2 as a result of homologous recombination between the endogenous gene encoding Apo-2 and altered genomic DNA encoding Apo-2 introduced into an embryonic cell of the animal. For example, cDNA encoding Apo-2 can be used to clone genomic DNA encoding Apo-2 in accordance with established techniques. A portion of the genomic DNA encoding Apo-2 can be deleted or replaced with another gene, such as a gene encoding a selectable

marker which can be used to monitor integration. Typically, several kilobases of unaltered flanking DNA (both at the 5' and 3' ends) are included in the vector [see e.g., Thomas and Capecchi, *Cell*, 51:503 (1987) for a description of homologous recombination vectors]. The vector is introduced into an embryonic stem cell line (e.g., by electroporation) and cells in which the introduced DNA has homologously recombined with the endogenous DNA are selected [see e.g., Li et al., *Cell*, 69:915 (1992)]. The selected cells are then injected into a blastocyst of an animal (e.g., a mouse or rat) to form aggregation chimeras [see e.g., Bradley, in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E. J. Robertson, ed. (IRL, Oxford, 1987), pp. 113-152]. A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term to create a "knock out" animal. Progeny harboring the homologously recombined DNA in their germ cells can be identified by standard techniques and used to breed animals in which all cells of the animal contain the homologously recombined DNA. Knockout animals can be characterized for instance, for their ability to defend against certain pathological conditions and for their development of pathological conditions due to absence of the Apo-2 polypeptide, including for example, development of tumors.

#### C. Anti-Apo-2 Antibody Preparation

The present invention further provides anti-Apo-2 antibodies. Antibodies against Apo-2 may be prepared as follows. Exemplary antibodies include polyclonal, monoclonal, humanized, bispecific, and heteroconjugate antibodies.

##### 1. Polyclonal Antibodies

The Apo-2 antibodies may comprise polyclonal antibodies. Methods of preparing polyclonal antibodies are known to the skilled artisan. Polyclonal antibodies can be raised in a mammal, for example, by one or more injections of an immunizing agent and, if desired, an adjuvant. Typically, the immunizing agent and/or adjuvant will be injected in the mammal by multiple subcutaneous or intraperitoneal injections. The immunizing agent may include the Apo-2 polypeptide or a fusion protein thereof. An example of a suitable immunizing agent is an Apo-2-IgG fusion protein, such as an Apo-2 ECD-IgG fusion protein. Cells expressing Apo-2 at their

surface may also be employed. It may be useful to conjugate the immunizing agent to a protein known to be immunogenic in the mammal being immunized. Examples of such immunogenic proteins which may be employed include but are not limited to keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, and soybean trypsin inhibitor. An aggregating agent such as alum may also be employed to enhance the mammal's immune response. Examples of adjuvants which may be employed include Freund's complete adjuvant and MPL-TDM adjuvant (monophosphoryl Lipid A, synthetic trehalose dicorynomycolate). The immunization protocol may be selected by one skilled in the art without undue experimentation. The mammal can then be bled, and the serum assayed for antibody titer. If desired, the mammal can be boosted until the antibody titer increases or plateaus.

## 2. Monoclonal Antibodies

The Apo-2 antibodies may, alternatively, be monoclonal antibodies. Monoclonal antibodies may be prepared using hybridoma methods, such as those described by Kohler and Milstein, supra. In a hybridoma method, a mouse, hamster, or other appropriate host animal, is typically immunized (such as described above) with an immunizing agent to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the immunizing agent. Alternatively, the lymphocytes may be immunized in vitro.

The immunizing agent will typically include the Apo-2 polypeptide or a fusion protein thereof. An example of a suitable immunizing agent is an Apo-2-IgG fusion protein or chimeric molecule. A specific example of an Apo-2 ECD-IgG immunogen is described in Example 9 below. Cells expressing Apo-2 at their surface may also be employed. Generally, either peripheral blood lymphocytes ("PBLs") are used if cells of human origin are desired, or spleen cells or lymph node cells are used if non-human mammalian sources are desired. The lymphocytes are then fused with an immortalized cell line using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell [Goding, Monoclonal Antibodies: Principles and Practice, Academic Press, (1986) pp. 59-103]. Immortalized cell lines are usually transformed mammalian cells, particularly myeloma cells of rodent, bovine and human origin. Usually, rat or mouse myeloma cell lines are employed.

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The hybridoma cells may be cultured in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, immortalized cells. For example, if the parental transformed cells lack the enzyme  
5 hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine ("HAT medium"), which substances prevent the growth of HGPRT-deficient cells.

Preferred immortalized cell lines are those that fuse  
10 efficiently, support stable high level expression of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. More preferred immortalized cell lines are murine myeloma lines, which can be obtained, for instance, from the Salk Institute Cell Distribution Center, San Diego, California  
15 and the American Type Culture Collection, Manassas, Virginia. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies [Kozbor, J. Immunol., 133:3001 (1984); Brodeur et al., Monoclonal Antibody Production Techniques and Applications, Marcel Dekker,  
20 Inc., New York, (1987) pp. 51-63].

The culture medium in which the hybridoma cells are cultured can then be assayed for the presence of monoclonal antibodies directed against Apo-2. Preferably, the binding specificity of monoclonal antibodies produced by the hybridoma  
25 cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA). Such techniques and assays are known in the art. The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson  
30 and Pollard, Anal. Biochem., 107:220 (1980).

After the desired hybridoma cells are identified, the clones may be subcloned by limiting dilution procedures and grown by standard methods [Goding, supra]. Suitable culture media for this purpose include, for example, Dulbecco's Modified Eagle's  
35 Medium and RPMI-1640 medium. Alternatively, the hybridoma cells may be grown *in vivo* as ascites in a mammal.

The monoclonal antibodies secreted by the subclones may be isolated or purified from the culture medium or ascites fluid by conventional immunoglobulin purification procedures such as, for



example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

The monoclonal antibodies may also be made by recombinant DNA methods, such as those described in U.S. Patent No. 4,816,567. DNA encoding the monoclonal antibodies of the invention can be readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells of the invention serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. The DNA also may be modified, for example, by substituting the coding sequence for human heavy and light chain constant domains in place of the homologous murine sequences [U.S. Patent No. 4,816,567; Morrison et al., supra] or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. Such a non-immunoglobulin polypeptide can be substituted for the constant domains of an antibody of the invention, or can be substituted for the variable domains of one antigen-combining site of an antibody of the invention to create a chimeric bivalent antibody.

As described in the Examples below, anti-Apo-2 monoclonal antibodies have been prepared. One of these antibodies, 3F11.39.7, has been deposited with ATCC and has been assigned deposit accession no. HB-12456. In one embodiment, the monoclonal antibodies of the invention will have the same biological characteristics as the monoclonal antibodies secreted by the hybridoma cell line(s) deposited under Accession No. HB-12456. The term "biological characteristics" is used to refer to the *in vitro* and/or *in vivo* activities or properties of the monoclonal antibody, such as the ability to specifically bind to Apo-2 or to substantially block, induce or enhance Apo-2 activation. As disclosed in the present specification, the 3F11.39.7 monoclonal antibody (HB-12456) is characterized as having agonistic activity for inducing apoptosis, binding to the Apo-2 receptor, having blocking activity as described in the Examples below, and having

some cross-reactivity to DR4 but not to DcR1 or DcR2. Optionally, the monoclonal antibody will bind to the same epitope as the 3F11.39.7 antibody disclosed herein. This can be determined by conducting various assays, such as described herein and in the Examples. For instance, to determine whether a monoclonal antibody has the same specificity as the 3F11.39.7 antibody specifically disclosed, one can compare activity in Apo-2 blocking and apoptosis induction assays, such as those described in the Examples below.

The antibodies of the invention may also comprise monovalent antibodies. Methods for preparing monovalent antibodies are well known in the art. For example, one method involves recombinant expression of immunoglobulin light chain and modified heavy chain. The heavy chain is truncated generally at any point in the Fc region so as to prevent heavy chain crosslinking. Alternatively, the relevant cysteine residues are substituted with another amino acid residue or are deleted so as to prevent crosslinking.

In vitro methods are also suitable for preparing monovalent antibodies. Digestion of antibodies to produce fragments thereof, particularly, Fab fragments, can be accomplished using routine techniques known in the art. For instance, digestion can be performed using papain. Examples of papain digestion are described in WO 94/29348 published 12/22/94 and U.S. Patent No. 4,342,566. Papain digestion of antibodies typically produces two identical antigen binding fragments, called Fab fragments, each with a single antigen binding site, and a residual Fc fragment. Pepsin treatment yields an F(ab')<sub>2</sub> fragment that has two antigen combining sites and is still capable of cross-linking antigen.

The Fab fragments produced in the antibody digestion also contain the constant domains of the light chain and the first constant domain (CH<sub>1</sub>) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH<sub>1</sub> domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. F(ab')<sub>2</sub> antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

### 3. Humanized Antibodies

The Apo-2 antibodies of the invention may further comprise humanized antibodies or human antibodies. Humanized forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab')<sub>2</sub>, or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin [Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature, 332:323-329 (1988); and Presta, Curr. Op. Struct. Biol., 2:593-596 (1992)].

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers [Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature, 332:323-327 (1988); Verhoeyen et al., Science, 239:1534-1536 (1988)], by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Patent No. 4,816,567),

wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues  
5 are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important in order to reduce antigenicity. According to the "best-  
10 fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody [Sims et al., J. Immunol., 151:2296 (1993);  
15 Chothia and Lesk, J. Mol. Biol., 196:901 (1987)]. Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies [Carter et al., Proc. Natl. Acad. Sci. USA,  
20 89:4285 (1992); Presta et al., J. Immunol., 151:2623 (1993)].

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of  
25 analysis of the parental sequences and various conceptual humanized products using three dimensional models of the parental and humanized sequences. Three dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display  
30 probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate  
35 immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the consensus and import sequence so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in

influencing antigen binding [see, WO 94/04679 published 3 March 1994].

Transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production can be employed. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge [see, e.g., Jakobovits et al., Proc. Natl. Acad. Sci. USA, 90:2551-255 (1993); Jakobovits et al., Nature, 362:255-258 (1993); Bruggemann et al., Year in Immuno., 7:33 (1993)].

Human antibodies can also be produced in phage display libraries [Hoogenboom and Winter, J. Mol. Biol., 227:381 (1992); Marks et al., J. Mol. Biol., 222:581 (1991)]. The techniques of Cole et al. and Boerner et al. are also available for the preparation of human monoclonal antibodies (Cole et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985) and Boerner et al., J. Immunol., 147(1):86-95 (1991)). Suitable methods for preparing phage libraries have been reviewed and are described in Winter et al., Annu. Rev. Immunol., 12:433-55 (1994); Soderlind et al., Immunological Reviews, 130:109-123 (1992); Hoogenboom, Tibtech February 1997, Vol. 15; Neri et al., Cell Biophysics, 27:47-61 (1995). Libraries of single chain antibodies may also be prepared by the methods described in WO 92/01047, WO 92/20791, WO 93/06213, WO 93/11236, WO 93/19172, WO 95/01438 and WO 95/15388. Antibody libraries are also commercially available, for example, from Cambridge Antibody Technologies (C.A.T.), Cambridge, UK. Binding selection against an antigen, in this case Apo-2, can be carried out as described in greater detail in the Examples below.

As described in the Examples below, anti-Apo-2 single-chain Fv (scFv) antibodies have been identified using a phage display library. Three of these antibodies, referred to herein as 16E2, 24C4 and 20E6, have been sequenced and characterized. The respective DNA and amino acid sequences and complementarity determining regions of these antibodies are shown in Figures 15A-15C and 16. In one embodiment of the invention, scFv Apo-2 antibodies will have the same biological characteristics as the 16E2, 24C4 or 20E6 antibodies identified herein. The term

"biological characteristics" is used to refer to the *in vitro* and/or *in vivo* activities or properties of the scFv antibody, such as the ability to specifically bind to Apo-2 or to substantially induce or enhance Apo-2 activation. As disclosed in the present specification, the 16E2, 24C4 and 20E6 antibodies are characterized as binding to Apo-2, having agonistic activity for inducing apoptosis, and having no cross-reactivity to DR4 or several of the other known molecules recognized by the Apo-2 ligand. Optionally, the scFv Apo-2 antibody will bind to the same epitope or epitopes recognized by the 16E2, 24C4 or 20E6 antibodies disclosed herein. This can be determined by conducting various assays, such as described herein and in the Examples. For instance, to determine whether a scFv antibody has the same specificity as the 16E2, 24C4 or 20E6 antibodies specifically disclosed, one can compare activity in apoptosis induction assays, such as those described in the Examples below.

Optionally the scFv antibodies to Apo-2 may include antibodies which contain a VH and VL chain that include one or more complementarity determining region (CDR) amino acid sequences identified in Figure 16 for the 16E2, 20E6, or 24C4 antibodies.

#### 4. Bispecific Antibodies

Bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens. In the present case, one of the binding specificities is for the Apo-2, the other one is for any other antigen, and preferably for a cell-surface protein or receptor or receptor subunit.

Methods for making bispecific antibodies are known in the art. Traditionally, the recombinant production of bispecific antibodies is based on the co-expression of two immunoglobulin heavy-chain/light-chain pairs, where the two heavy chains have different specificities [Milstein and Cuello, Nature, 305:537-539 (1983)]. Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of ten different antibody molecules, of which only one has the correct bispecific structure. The purification of the correct molecule is usually accomplished by affinity chromatography steps.

Similar procedures are disclosed in WO 93/08829, published 13 May 1993, and in Traunecker et al., EMBO J., 10:3655-3659 (1991).

According to a different and more preferred approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy-chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light-chain binding present in at least one of the fusions. DNAs encoding the immunoglobulin heavy-chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance. In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy-chain/light-chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690 published 3 March 1994. For further details of generating bispecific antibodies see, for example, Suresh et al., Methods in Enzymology, 121:210 (1986).

#### 35 5. Heteroconjugate Antibodies

Heteroconjugate antibodies are also within the scope of the present invention. Heteroconjugate antibodies are composed of two covalently joined antibodies. Such antibodies have, for example, been proposed to target immune system cells to unwanted

cells [US Patent No. 4,676,980], and for treatment of HIV infection [WO 91/00360; WO 92/200373; EP 03089]. It is contemplated that the antibodies may be prepared in vitro using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins may be constructed using a disulfide exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptobutyrimidate and those disclosed, for example, in U.S. Pat. No. 4,676,980.

#### 6. Triabodies

Triabodies are also within the scope of the invention. Such antibodies are described for instance in Iliades et al., FEBS Letters, 409:437-441 (1997) and Korrt et al., Protein Engineering, 10:423-433 (1997).

#### 7. Other Modifications

Other modifications of the Apo-2 antibodies are contemplated. For example, it may be desirable to modify the antibodies of the invention with respect to effector function, so as to enhance the therapeutic effectiveness of the antibodies. For instance, cysteine residue(s) may be introduced into the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing [see, e.g., Caron et al., J. Exp. Med., 176:1191-1195 (1992); Shopes, J. Immunol., 148:2918-2922 (1992). Homodimeric antibodies may also be prepared using heterobifunctional cross-linkers as described in Wolff et al., Cancer Research, 53:2560-2565 (1993). Ghetie et al., Proc. Natl. Acad. Sci., 94:7509-7514 (1997), further describe preparation of IgG-IgG homodimers and disclose that such homodimers can enhance apoptotic activity as compared to the monomers. Alternatively, the antibodies can be engineered to have dual Fc regions [see, Stevenson et al., Anti-Cancer Drug Design, 3:219-230 (1989)].

It may be desirable to modify the amino acid sequences of the antibodies disclosed herein. Sequences within the scFv complementary determining or linker regions (as shown in Figure 16) may be modified for instance to modulate the biological activities of these antibodies. Variations in the full-length scFv sequence or in various domains of the scFv molecules described herein, can be



made, for example, using any of the techniques and guidelines for conservative and non-conservative mutations set forth, for instance, in U.S. Pat. No. 5,364,934. Variations may be a substitution, deletion or insertion of one or more codons encoding a scFv that results in a change in the amino acid sequence of the scFv as compared with the native sequence scFv. Optionally, the variation is by substitution of at least one amino acid with any other amino acid in one or more of the domains of the scFv molecule. The variations can be made using methods known in the art such as oligonucleotide-mediated (site-directed) mutagenesis, alanine scanning, and PCR mutagenesis. Site-directed mutagenesis [Carter et al., Nucl. Acids Res., 13:4331 (1986); Zoller et al., Nucl. Acids Res., 10:6487 (1987)], cassette mutagenesis [Wells et al., Gene, 34:315 (1985)], restriction selection mutagenesis [Wells et al., Philos. Trans. R. Soc. London SerA, 317:415 (1986)] or other known techniques can be performed on the cloned DNA to produce the scFv variant DNA.

The antibodies may optionally be covalently attached or conjugated to one or more chemical groups. A polyol, for example, can be conjugated to an antibody molecule at one or more amino acid residues, including lysine residues as disclosed in WO 93/00109. Optionally, the polyol is a poly(alkylene glycol), such as poly(ethylene glycol) (PEG), however, those skilled in the art recognize that other polyols, such as, for example, poly(propylene glycol) and polyethylene-polypropylene glycol copolymers, can be employed using techniques for conjugating PEG to polypeptides. A variety of methods for pegylating polypeptides have been described. See, e.g. U.S. Patent No. 4,179,337 which discloses the conjugation of a number of hormones and enzymes to PEG and polypropylene glycol to produce physiologically active compositions having reduced immunogenicities.

The antibodies may also be fused or linked to another heterologous polypeptide or amino acid sequence such as an epitope tag. Epitope tag polypeptides and methods of their use are described above in Section A, paragraph 8. Any of the tags described herein may be linked to the antibodies. The Examples below, for instance, describe His-tagged and gD-tagged single-chain antibodies.

#### D. Therapeutic Uses for Apo-2 Antibodies

The Apo-2 antibodies of the invention have therapeutic utility. Agonistic Apo-2 antibodies, for instance, may be employed to activate or stimulate apoptosis in cancer cells. Accordingly,  
5 the invention provides methods for treating cancer using such Apo-2 antibodies. It is of course contemplated that the methods of the invention can be employed in combination with still other therapeutic techniques such as surgery.

The agonist is preferably administered to the mammal in  
10 a carrier. Suitable carriers and their formulations are described in Remington's Pharmaceutical Sciences, 16th ed., 1980, Mack Publishing Co., edited by Oslo et al. Typically, an appropriate amount of a pharmaceutically-acceptable salt is used in the formulation to render the formulation isotonic. Examples of a  
15 pharmaceutically-acceptable carrier include saline, Ringer's solution and dextrose solution. The pH of the solution is preferably from about 5 to about 8, and more preferably from about 7 to about 7.5. Further carriers include sustained release preparations such as semipermeable matrices of solid hydrophobic  
20 polymers containing the agonist, which matrices are in the form of shaped articles, e.g., films, liposomes or microparticles. It will be apparent to those persons skilled in the art that certain carriers may be more preferable depending upon, for instance, the route of administration and concentration of agonist being  
25 administered.

The agonist antibody can be administered to the mammal by injection (e.g., intravenous, intraperitoneal, subcutaneous, intramuscular), or by other methods such as infusion that ensure its delivery to the bloodstream in an effective form. The agonist  
30 may also be administered by intratumoral, peritumoral, intralesional, or perilesional routes, to exert local as well as systemic therapeutic effects. Local or intravenous injection is preferred.

Effective dosages and schedules for administering the  
35 agonist antibody may be determined empirically, and making such determinations is within the skill in the art. Those skilled in the art will understand that the dosage of agonist that must be administered will vary depending on, for example, the mammal which will receive the agonist, the route of administration, the

particular type of agonist used and other drugs being administered to the mammal. Guidance in selecting appropriate doses for antibody agonists is found in the literature on therapeutic uses of antibodies, e.g., Handbook of Monoclonal Antibodies, Ferrone et al., eds., Noyes Publications, Park Ridge, N.J., (1985) ch. 22 and pp. 303-357; Smith et al., Antibodies in Human Diagnosis and Therapy, Haber et al., eds., Raven Press, New York (1977) pp. 365-389. A typical daily dosage of the agonist used alone might range from about 1 µg/kg to up to 100 mg/kg of body weight or more per day, depending on the factors mentioned above.

The agonist antibody may also be administered to the mammal in combination with effective amounts of one or more other therapeutic agents or in conjunction with radiation treatment. Therapeutic agents contemplated include chemotherapeutics as well as immunoadjuvants and cytokines. Chemotherapies contemplated by the invention include chemical substances or drugs which are known in the art and are commercially available, such as Doxorubicin, 5-Fluorouracil, Cytosine arabinoside ("Ara-C"), Cyclophosphamide, Thiotepa, Busulfan, Cytosine, Taxol, Methotrexate, Cisplatin, Melphalan, Vinblastine and Carboplatin. The agonist may be administered sequentially or concurrently with the one or more other therapeutic agents. The amounts of agonist and therapeutic agent depend, for example, on what type of drugs are used, the cancer being treated, and the scheduling and routes of administration but would generally be less than if each were used individually.

Following administration of agonist to the mammal, the mammal's cancer and physiological condition can be monitored in various ways well known to the skilled practitioner. For instance, tumor mass may be observed physically or by standard x-ray imaging techniques.

The Apo-2 antibodies of the invention may also be useful in enhancing immune-mediated cell death in cells expressing Apo-2, for instance, through complement fixation or ADCC. Alternatively, antagonistic antibodies may be used to block excessive apoptosis (for instance in neurodegenerative disease) or to block potential autoimmune/inflammatory effects of Apo-2 resulting from NF-κB

activation. Such antagonistic antibodies can be utilized according to the therapeutic methods and techniques described above.

E. Non-therapeutic Uses for Apo-2 Antibodies

Apo-2 antibodies may further be used in diagnostic assays for Apo-2, e.g., detecting its expression in specific cells, tissues, or serum. Various diagnostic assay techniques known in the art may be used, such as competitive binding assays, direct or indirect sandwich assays and immunoprecipitation assays conducted in either heterogeneous or homogeneous phases [Zola, Monoclonal Antibodies: A Manual of Techniques, CRC Press, Inc. (1987) pp. 147-158]. The antibodies used in the diagnostic assays can be labeled with a detectable moiety. The detectable moiety should be capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety may be a radioisotope, such as  $^3\text{H}$ ,  $^{14}\text{C}$ ,  $^{32}\text{P}$ ,  $^{35}\text{S}$ , or  $^{125}\text{I}$ , a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin, or an enzyme, such as alkaline phosphatase, beta-galactosidase or horseradish peroxidase. Any method known in the art for conjugating the antibody to the detectable moiety may be employed, including those methods described by Hunter et al., Nature, 144:945 (1962); David et al., Biochemistry, 13:1014 (1974); Pain et al., J. Immunol. Meth., 40:219 (1981); and Nygren, J. Histochem. and Cytochem., 30:407 (1982).

Apo-2 antibodies also are useful for the affinity purification of Apo-2 from recombinant cell culture or natural sources. In this process, the antibodies against Apo-2 are immobilized on a suitable support, such as Sephadex resin or filter paper, using methods well known in the art. The immobilized antibody then is contacted with a sample containing the Apo-2 to be purified, and thereafter the support is washed with a suitable solvent that will remove substantially all the material in the sample except the Apo-2, which is bound to the immobilized antibody. Finally, the support is washed with another suitable solvent that will release the Apo-2 from the antibody.

35

F. Kits Containing Apo-2 or Apo-2 Antibodies

In a further embodiment of the invention, there are provided articles of manufacture and kits containing Apo-2 or Apo-2 antibodies which can be used, for instance, for the therapeutic or

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non-therapeutic applications described above. The article of manufacture comprises a container with a label. Suitable containers include, for example, bottles, vials, and test tubes. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which includes an active agent that is effective for therapeutic or non-therapeutic applications, such as described above. The active agent in the composition is Apo-2 or an Apo-2 antibody. The label on the container indicates that the composition is used for a specific therapy or non-therapeutic application, and may also indicate directions for either *in vivo* or *in vitro* use, such as those described above.

The kit of the invention will typically comprise the container described above and one or more other containers comprising materials desirable from a commercial and user standpoint, including buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

\*\*\*\*\*

The following examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

All patent and literature references cited in the present specification are hereby incorporated by reference in their entirety.

25

#### EXAMPLES

All restriction enzymes referred to in the examples were purchased from New England Biolabs and used according to manufacturer's instructions. All other commercially available reagents referred to in the examples were used according to manufacturer's instructions unless otherwise indicated. The source of those cells identified in the following examples, and throughout the specification, by ATCC accession numbers is the American Type Culture Collection, Manassas, VA.

35

#### EXAMPLE 1

##### Isolation of cDNA clones Encoding Human Apo-2

Expressed sequence tag (EST) DNA databases (LIFESEQ™, Incyte Pharmaceuticals, Palo Alto, CA) were searched and an EST was

identified which showed homology to the death domain of the Apo-3 receptor [Marsters et al., Curr. Biol., 6:750 (1996)]. Human pancreas and kidney lgt10 bacteriophage cDNA libraries (both purchased from Clontech) were ligated into pRK5 vectors as follows.

- 5 Reagents were added together and incubated at 16°C for 16 hours: 5X T4 ligase buffer (3 ml); pRK5, Xho1, Not1 digested vector, 0.5 mg, 1 ml); cDNA (5 ml) and distilled water (6 ml). Subsequently, additional distilled water (70 ml) and 10 mg/ml tRNA (0.1 ml) were added and the entire reaction was extracted through
- 10 phenol:chloroform:isoamyl alcohol (25:24:1). The aqueous phase was removed, collected and diluted into 5M NaCl (10 ml) and absolute ethanol (-20°C, 250 ml). This was then centrifuged for 20 minutes at 14,000 x g, decanted, and the pellet resuspended into 70% ethanol (0.5 ml) and centrifuged again for 2 minutes at 14,000 x g.
- 15 The DNA pellet was then dried in a speedvac and eluted into distilled water (3 ml) for use in the subsequent procedure.

- The ligated cDNA/pRK5 vector DNA prepared previously was chilled on ice to which was added electrocompetent DH10B bacteria (Life Tech., 20 ml). The bacteria vector mixture was then
- 20 electroporated as per the manufacturers recommendation. Subsequently SOC media (1 ml) was added and the mixture was incubated at 37°C for 30 minutes. The transformants were then plated onto 20 standard 150 mm LB plates containing ampicillin and incubated for 16 hours (37°C) to allow the colonies to grow.
- 25 Positive colonies were then scraped off and the DNA isolated from the bacterial pellet using standard CsCl-gradient protocols.

- An enriched 5'-cDNA library was then constructed to obtain a bias of cDNA fragments which preferentially represents the 5' ends of cDNA's contained within the library. 10 mg of the
- 30 pooled isolated full-length library plasmid DNA (41 ml) was combined with Not 1 restriction buffer (New England Biolabs, 5 ml) and Not 1 (New England Biolabs, 4 ml) and incubated at 37°C for one hour. The reaction was extracted through phenol:chloroform:isoamyl alcohol (25:24:1, 50 ml), the aqueous phase removed, collected and
- 35 resuspended into 5M NaCl (5 ml) and absolute ethanol (-20°C, 150 ml). This was then centrifuged for 20 minutes at 14,000 x g, decanted, resuspended into 70% ethanol (0.5 ml) and centrifuged again for 2 minutes at 14,000 x g. The supernatant was then

removed, the pellet dried in a speedvac and resuspended in distilled water (10 ml).

The following reagents were brought together and incubated at 37°C for 2 hours: distilled water (3 ml); linearized DNA library (1 mg, 1 ml); Ribonucleotide mix (Invitrogen, 10 ml); transcription buffer (Invitrogen, 2 ml) and Sp6 enzyme mix. The reaction was then extracted through phenol:chloroform:isoamyl alcohol (25:24:1, 50 ml) and the aqueous phase was removed, collected and resuspended into 5M NaCl (5 ml) and absolute ethanol (-20°C, 150 ml) and centrifuged for 20 minutes at 14,000 x g. The pellet was then decanted and resuspended in 70% ethanol (0.5 ml), centrifuged again for 2 minutes at 14,000 x g, decanted, dried in a speedvac and resuspended into distilled water (10 ml).

The following reagents were added together and incubated at 16°C for 16 hours: 5X T4 ligase buffer (Life Tech., 3 ml); pRK5 Cla-Sal digested vector, 0.5 mg, 1 ml); cDNA (5 ml); distilled water (6 ml). Subsequently, additional distilled water (70 ml) and 10 mg/ml tRNA (0.1 ml) was added and the entire reaction was extracted through phenol:chloroform:isoamyl alcohol (25:24:1, 100 ml). The aqueous phase was removed, collected and diluted by 5M NaCl (10 ml) and absolute ethanol (-20°C, 250 ml) and centrifuged for 20 minutes at 14,000 x g. The DNA pellet was decanted, resuspended into 70% ethanol (0.5 ml) and centrifuged again for 2 minutes at 14,000 x g. The supernatant was removed and the residue pellet was dried in a speedvac and resuspended in distilled water (3 ml). The ligated cDNA/pSST-amy.1 vector DNA was chilled on ice to which was added electrocompetent DH10B bacteria (Life Tech., 20 ml). The bacteria vector mixture was then electroporated as recommended by the manufacturer. Subsequently, SOC media (Life Tech., 1 ml) was added and the mixture was incubated at 37°C for 30 minutes. The transformants were then plated onto 20 standard 150 mm LB plates containing ampicillin and incubated for 16 hours (37°C). Positive colonies were scraped off the plates and the DNA was isolated from the bacterial pellet using standard protocols, e.g. CsCl-gradient.

The cDNA libraries were screened by hybridization with a synthetic oligonucleotide probe:

GGGAGCCGCTCATGAGGAAGTTGGGCCTCATGGACAATGAGATAAAGGTGGCTAAAGCTGAGGCAGC  
GGG (SEQ ID NO:3) based on the EST.

Three cDNA clones were sequenced in entirety. The overlapping coding regions of the cDNAs were identical except for  
5 codon 410 (using the numbering system for Fig. 1); this position encoded a leucine residue (TTG) in both pancreatic cDNAs, and a methionine residue (ATG) in the kidney cDNA, possibly due to polymorphism.

The entire nucleotide sequence of Apo-2 is shown in  
10 Figure 1 (SEQ ID NO:2). Clone 27868 (also referred to as pRK5-Apo-2 deposited as ATCC 209021, as indicated below) contains a single open reading frame with an apparent translational initiation site at nucleotide positions 140-142 [Kozak et al., supra] and ending at the stop codon found at nucleotide positions 1373-1375 (Fig. 1; SEQ  
15 ID NO:2). The predicted polypeptide precursor is 411 amino acids long, a type I transmembrane protein, and has a calculated molecular weight of approximately 45 kDa. Hydropathy analysis (not shown) suggested the presence of a signal sequence (residues 1-53), followed by an extracellular domain (residues 54-182), a  
20 transmembrane domain (residues 183-208), and an intracellular domain (residues 209-411) (Fig. 2A; SEQ ID NO:1). N-terminal amino acid sequence analysis of Apo-2-IgG expressed in 293 cells showed that the mature polypeptide starts at amino acid residue 54, indicating that the actual signal sequence comprises residues 1-53.  
25 Apo-2 polypeptide is obtained or obtainable by expressing the molecule encoded by the cDNA insert of the deposited ATCC 209021 vector.

TNF receptor family proteins are typically characterized by the presence of multiple (usually four) cysteine-rich domains in  
30 their extracellular regions -- each cysteine-rich domain being approximately 45 amino acids long and containing approximately 6, regularly spaced, cysteine residues. Based on the crystal structure of the type 1 TNF receptor, the cysteines in each domain typically form three disulfide bonds in which usually cysteines 1  
35 and 2, 3 and 5, and 4 and 6 are paired together. Like DR4, Apo-2 contains two extracellular cysteine-rich pseudorepeats (Fig. 2A), whereas other identified mammalian TNFR family members contain three or more such domains [Smith et al., Cell, 76:959 (1994)].



The cytoplasmic region of Apo-2 contains a death domain (amino acid residues 324-391 shown in Fig. 1; see also Fig. 2A) which shows significantly more amino acid sequence identity to the death domain of DR4 (64%) than to the death domain of TNFR1 (30%); CD95 (19%); or Apo-3/DR3 (29%) (Fig. 2B). Four out of six death domain amino acids that are required for signaling by TNFR1 [Tartaglia et al., *supra*] are conserved in Apo-2 while the other two residues are semi-conserved (see Fig. 2B).

Based on an alignment analysis (using the ALIGN™ computer program) of the full-length sequence, Apo-2 shows more sequence identity to DR4 (55%) than to other apoptosis-linked receptors, such as TNFR1 (19%); CD95 (17%); or Apo-3 (also referred to as DR3, WSL-1 or TRAMP) (29%).

## EXAMPLE 2

### A. Expression of Apo-2 ECD

A soluble extracellular domain (ECD) fusion construct was prepared. An Apo-2 ECD (amino acid residues 1-184 shown in Figure 1) was obtained by PCR and fused to a C-terminal Flag epitope tag (Sigma). (The Apo-2 ECD construct included residues 183 and 184 shown in Figure 1 to provide flexibility at the junction, even though residues 183 and 184 are predicted to be in the transmembrane region). The Flag epitope-tagged molecule was then inserted into pRK5, and expressed by transient transfection into human 293 cells (ATCC CRL 1573).

After a 48 hour incubation, the cell supernatants were collected and either used directly for co-precipitation studies (see Example 3) or subjected to purification of the Apo-2 ECD-Flag by affinity chromatography on anti-Flag agarose beads, according to manufacturer's instructions (Sigma).

### B. Expression of Apo-2 ECD as an Immunoadhesin

A soluble Apo-2 ECD immunoadhesin construct was prepared. The Apo-2 ECD (amino acids 1-184 shown in Fig. 1) was fused to the hinge and Fc region of human immunoglobulin G<sub>1</sub> heavy chain in pRK5 as described previously [Ashkenazi et al., *Proc. Natl. Acad. Sci.*, 88:10535-10539 (1991)]. The immunoadhesin was expressed by transient transfection into human 293 cells and

purified from cell supernatants by protein A affinity chromatography, as described by Ashkenazi et al., supra.

### EXAMPLE 3

#### 5           Immunoprecipitation Assay Showing Binding Interaction             Between Apo-2 and Apo-2 Ligand

To determine whether Apo-2 and Apo-2L interact or associate with each other, supernatants from mock-transfected 293 cells or from 293 cells transfected with Apo-2 ECD-Flag (described  
10 in Example 2 above) (5 ml) were incubated with 5 µg poly-histidine-tagged soluble Apo-2L [Pitti et al., supra] for 30 minutes at room temperature and then analyzed for complex formation by a co-precipitation assay.

The samples were subjected to immunoprecipitation using  
15 25 µl anti-Flag conjugated agarose beads (Sigma) or Nickel-conjugated agarose beads (Qiagen). After a 1.5 hour incubation at 4° C, the beads were spun down and washed four times in phosphate buffered saline (PBS). By using anti-Flag agarose, the Apo-2L was precipitated through the Flag-tagged Apo-2 ECD; by using Nickel-  
20 agarose, the Apo-2 ECD was precipitated through the His-tagged Apo-2L. The precipitated proteins were released by boiling the beads for 5 minutes in SDS-PAGE buffer, resolved by electrophoresis on 12% polyacrylamide gels, and then detected by immunoblot with anti-Apo-2L or anti-Flag antibody (2 µg/ml) as described in Marsters et  
25 al., J. Biol. Chem., (1997).

The results, shown in Figure 3, indicate that the Apo-2 ECD and Apo-2L can associate with each other.

The binding interaction was further analyzed by purifying Apo-2 ECD from the transfected 293 cell supernatants with  
30 anti-Flag beads (see Example 2) and then analyzing the samples on a BIACORE™ instrument. The BIACORE™ analysis indicated a dissociation constant ( $K_d$ ) of about 1 nM. BIACORE™ analysis also showed that the Apo-2 ECD is not capable of binding other apoptosis-inducing TNF family members, namely, TNF-alpha  
35 (Genentech, Inc., Pennica et al., Nature, 312:712 (1984), lymphotoxin-alpha (Genentech, Inc.), or Fas/Apo-1 ligand (Alexis Biochemicals). The data thus shows that Apo-2 is a specific receptor for Apo-2L.

EXAMPLE 4Induction of Apoptosis by Apo-2

Because death domains can function as oligomerization  
 5 interfaces, over-expression of receptors that contain death domains  
 may lead to activation of signaling in the absence of ligand  
 [Frazer et al., supra, Nagata et al., supra]. To determine whether  
 Apo-2 was capable of inducing cell death, human 293 cells or HeLa  
 cells (ATCC CCL 2.2) were transiently transfected by calcium  
 10 phosphate precipitation (293 cells) or electroporation (HeLa cells)  
 with a pRK5 vector or pRK5-based plasmids encoding Apo-2 and/or  
 CrmA. When applicable, the total amount of plasmid DNA was  
 adjusted by adding vector DNA. Apoptosis was assessed 24 hours  
 after transfection by morphology (Fig. 4A); DNA fragmentation (Fig.  
 15 4B); or by FACS analysis of phosphatidylserine exposure (Fig. 4C)  
 as described in Marsters et al., Curr. Biol., 6:1669 (1996). As  
 shown in Figs. 4A and 4B, the Apo-2 transfected 293 cells underwent  
 marked apoptosis.

For samples assayed by FACS, the HeLa cells were co-  
 20 transfected with pRK5-CD4 as a marker for transfection and  
 apoptosis was determined in CD4-expressing cells; FADD was co-  
 transfected with the Apo-2 plasmid; the data are means  $\pm$  SEM of at  
 least three experiments, as described in Marsters et al., Curr.  
Biol., 6:1669 (1996). The caspase inhibitors, DEVD-fmk (Enzyme  
 25 Systems) or z-VAD-fmk (Research Biochemicals Intl.) were added at  
 200  $\mu$ M at the time of transfection. As shown in Fig. 4C, the  
 caspase inhibitors CrmA, DEVD-fmk, and z-VAD-fmk blocked apoptosis  
 induction by Apo-2, indicating the involvement of Ced-3-like  
 proteases in this response.

30 FADD is an adaptor protein that mediates apoptosis  
 activation by CD95, TNFR1, and Apo-3/DR3 [Nagata et al., supra],  
 but does not appear necessary for apoptosis induction by Apo-2L  
 [Marsters et al., supra] or by DR4 [Pan et al., supra]. A  
 dominant-negative mutant form of FADD, which blocks apoptosis  
 35 induction by CD95, TNFR1, or Apo-3/DR3 [Frazer et al., supra;  
 Nagata et al., supra; Chinnayian et al., supra] did not inhibit  
 apoptosis induction by Apo-2 when co-transfected into HeLa cells  
 with Apo-2 (Fig. 4C). These results suggest that Apo-2 signals  
 apoptosis independently of FADD. Consistent with this conclusion,

a glutathione-S-transferase fusion protein containing the Apo-2 cytoplasmic region did not bind to *in vitro* transcribed and translated FADD (data not shown).

5

#### EXAMPLE 5

##### Inhibition of Apo-2L Activity by Soluble Apo-2 ECD

Soluble Apo-2L (0.5 µg/ml, prepared as described in Pitti et al., supra) was pre-incubated for 1 hour at room temperature with PBS buffer or affinity-purified Apo-2 ECD (5  
10 µg/ml) together with anti-Flag antibody (Sigma) (1 µg/ml) and added to HeLa cells. After a 5 hour incubation, the cells were analyzed for apoptosis by FACS (as above) (Fig. 4D).

Apo-2L induced marked apoptosis in HeLa cells, and the soluble Apo-2 ECD was capable of blocking Apo-2L action (Fig. 4D),  
15 confirming a specific interaction between Apo-2L and Apo-2. Similar results were obtained with the Apo-2 ECD immunoadhesin (Fig. 4D). Dose-response analysis showed half-maximal inhibition at approximately 0.3 nM Apo-2 immunoadhesin (Fig. 4E).

20

#### EXAMPLE 6

##### Activation of NF-κB by Apo-2

An assay was conducted to determine whether Apo-2 activates NF-κB.

HeLa cells were transfected with pRK5 expression  
25 plasmids encoding full-length native sequence Apo-2, DR4 or Apo-3 and harvested 24 hours after transfection. Nuclear extracts were prepared and 1 µg of nuclear protein was reacted with a <sup>32</sup>P-labelled NF-κB-specific synthetic oligonucleotide probe  
ATCAGGGACTTTCCGCTGGGGACTTTCCG (SEQ ID NO:4) [see, also, MacKay et  
30 al., J. Immunol., 153:5274-5284 (1994)], alone or together with a 50-fold excess of unlabelled probe, or with an irrelevant <sup>32</sup>P-labelled synthetic oligonucleotide  
AGGATGGGAAGTGTGTGATATATCCTTGAT (SEQ ID NO:5). In some samples, antibody to p65/RelA subunits of NF-κB (1 µg/ml; Santa Cruz  
35 Biotechnology) was added. DNA binding was analyzed by an electrophoretic mobility shift assay as described by Hsu et al., supra; Marsters et al., supra, and MacKay et al., supra.

The results are shown in Fig. 5C. Both ALLN and cyclohexamide increased the level of Apo-2L-induced apoptosis in the HeLa cells. The data indicates that Apo-2L can induce protective NF- $\kappa$ B-dependent genes. The data also indicates that Apo-2L is capable of activating NF- $\kappa$ B in certain cell lines and that both Apo-2 and DR4 may mediate that function.

- 70 -

4.6 kilobase <sup>32</sup>P-labelled DNA probe based on the full length Apo-2 cDNA; the probe was generated by digesting the pRK5-Apo-2 plasmid with EcoRI. Human fetal RNA blot MTN (Clontech), human adult RNA blot MTN-II (Clontech), and human cancer cell line RNA blot (Clontech) were incubated with the DNA probes. Blots were incubated with the probes in hybridization buffer (5X SSPE; 2X Denhardt's solution; 100 mg/mL denatured sheared salmon sperm DNA; 50% formamide; 2% SDS) for 60 hours at 42°C. The blots were washed several times in 2X SSC; 0.05% SDS for 1 hour at room temperature, followed by a 30 minute wash in 0.1X SSC; 0.1% SDS at 50°C. The blots were developed after overnight exposure.

As shown in Fig. 6A, a predominant mRNA transcript of approximately 4.6kb was detected in multiple tissues. Expression was relatively high in fetal and adult liver and lung, and in adult ovary and peripheral blood leukocytes (PBL), while no mRNA expression was detected in fetal and adult brain. Intermediate levels of expression were seen in adult colon, small intestine, testis, prostate, thymus, pancreas, kidney, skeletal muscle, placenta, and heart. Several adult tissues that express Apo-2, e.g., PBL, ovary, and spleen, have been shown previously to express DR4 [Pan et al., *supra*], however, the relative levels of expression of each receptor mRNA appear to be different.

As shown in Fig. 6B, Apo-2 mRNA was expressed relatively high in 6 of 8 human cancer cell lines examined, namely, HL60 promyelocytic leukemia, HeLa S3 cervical carcinoma, K562 chronic myelogenous leukemia, SW 480 colorectal adenocarcinoma, A549 lung carcinoma, and G361 melanoma. There was also detectable expression in Burkitt's lymphoma (Raji) cells. Thus, Apo-2 may be useful as a target for inducing apoptosis in cancer cells from lymphoid as well as non-lymphoid tumors.

#### B. In Situ Hybridization

Expression of Apo-2 in normal and in cancerous human tissues was examined by *in situ* hybridization. In addition, several different chimp and rhesus monkey tissues were examined for Apo-2 expression. These tissues included: human fetal tissues (E12-E16 weeks) - placenta, umbilical cord, liver, kidney, adrenal gland, thyroid, lung, heart, great vessels, esophagus, stomach, small intestine, spleen, thymus, pancreas, brain, eye, spinal cord,

body wall, pelvis and lower limb; adult human tissues - kidney, bladder, adrenal gland, spleen, lymph node, pancreas, lung, skin, retina, liver; chimp tissues - salivary gland, stomach, thyroid, parathyroid, tongue, thymus, ovary, lymph node, and peripheral  
 5 nerve; rhesus monkey tissues - cerebral cortex, hippocampus, cerebellum and penis; human tumor tissue - lung adenocarcinoma, testis, lung carcinoma, breast carcinoma, fibroadenoma, soft tissue sarcoma.

Tissue samples were paraffin-embedded and sectioned.  
 10 Later, the sectioned tissues were deparaffinized and the slides placed in water. The slides were rinsed twice for five minutes at room temperature in 2X SSC. After rinsing, the slides were placed in 20 µg/ml proteinase K (in Rnase-free buffer) for 15 minutes at 37 °C (for fetal tissues) or 8X proteinase K for 30 minutes at 37 °C  
 15 (for formalin tissues). The slides were then rinsed again in 0.5X SSC and dehydrated. Prior to hybridization, the slides were placed in a plastic box lined with buffer (4X SSC, 50% formamide)-saturated filter paper. The tissues were covered with 50 µl hybridization buffer (3.75g Dextran sulfate plus 6 ml water;  
 20 vortexed and heated for 2 minutes; cooled on ice and 18.75 ml formamide, 3.75 ml 20X SSC and 9 ml water added) and incubated at 42 °C for 1 to 4 hours.

Hybridization was conducted using a <sup>33</sup>P-labelled probe consisting of nucleotides 706-1259 of SEQ ID NO:2. The probe was  
 25 added to the slides in hybridization buffer and incubated overnight at 55 °C. Multiple washing steps were then performed sequentially as follows: twice for 10 minutes at room temperature in 2X SSC, EDTA buffer (400 ml 20X SSC, 16 ml 0.25M EDTA); once for 30 minutes at 37 °C in 20 µg/ml RNase A; twice for 10 minutes at room  
 30 temperature in 2X SSC, EDTA buffer; once for 2 hours at 55 °C in 0.1X SSC, EDTA buffer; twice for 10 minutes at room temperature in 0.5X SSC. Dehydration was performed for 2 minutes each in 50%, 70%, 90% EtOH containing 0.3 M NH<sub>4</sub>AC. Finally, the slides were air-dried for 2 hours and exposed to film.

35 Expression of Apo-2 in the fetal tissues appeared strongest over hepatocytes in liver, developing glomeruli in kidney, adrenal cortex, and epithelium of gastrointestinal tract. Moderate expression was observed over epithelial cells in lung and

at sites of vascularization of a bone growth plate. A relatively low level expression was observed over thyroid epithelial cells and cells in cardiac ventricles. Expression was observed over lymphoid cells in the thymic medulla, developing lymph glands and placenta cytotrophoblast cells.

Expression of Apo-2 in adult tissues was observed over resting oocytes in primordial follicles and low levels over granulosa cells of developing follicles in chimp ovary. Expression was observed in cirrhotic livers over hepatocytes at the edge of nodules (i.e., area of damage, normal adult liver was negative). Other tissues were negative for expression.

In the cancer tissues examined, Apo-2 expression was found in two lung adenocarcinomas and two germ cell tumors of the testis. Two additional lung carcinomas (one squamous) were negative. One of five breast carcinomas was positive (there was expression in normal breast tissue). In a fibroadenoma, there appeared to be expression over both epithelial and stromal elements. A soft tissue sarcoma was also positive. Other tissues examined were negative.

#### EXAMPLE 8

##### Chromosomal Localization of the Apo-2 gene

Chromosomal localization of the human Apo-2 gene was examined by radiation hybrid (RH) panel analysis. RH mapping was performed by PCR using a human-mouse cell radiation hybrid panel (Research Genetics) and primers based on the coding region of the Apo-2 cDNA [Gelb et al., Hum. Genet., 98:141 (1996)]. Analysis of the PCR data using the Stanford Human Genome Center Database indicates that Apo-2 is linked to the marker D8S481, with an LOD of 11.05; D8S481 is linked in turn to D8S2055, which maps to human chromosome 8p21. A similar analysis of DR4 showed that DR4 is linked to the marker D8S2127 (with an LOD of 13.00), which maps also to human chromosome 8p21.

To Applicants' present knowledge, to date, no other member of the TNFR gene family has been located to chromosome 8.



EXAMPLE 9Preparation of Monoclonal Antibodies Specific for Apo-2

Balb/c mice (obtained from Charles River Laboratories)

5 were immunized by injecting 0.5µg/50µl of an Apo-2 ECD immunoadhesin protein (diluted in MPL-TDM adjuvant purchased from Ribi Immunochemical Research Inc., Hamilton, MT) 11 times into each hind foot pad at 3-4 day intervals. The Apo-2 ECD immunoadhesin protein was generated by fusing an extracellular domain sequence of Apo-2  
10 (amino acids 1-184 shown in Fig. 1) to the hinge and Fc region of human immunoglobulin G<sub>1</sub> heavy chain in pRK5 as described previously [Ashkenazi et al., Proc. Natl. Acad. Sci., 88:10535-10539 (1991)]. The immunoadhesin protein was expressed by transient transfection into human 293 cells and purified from cell supernatants by protein  
15 A affinity chromatography, as described by Ashkenazi et al., supra (See also Example 2B above).

Three days after the final boost, popliteal lymph nodes were removed from the mice and a single cell suspension was prepared in DMEM media (obtained from Biowhitakker Corp.)  
20 supplemented with 1% penicillin-streptomycin. The lymph node cells were then fused with murine myeloma cells P3X63AgU.1 (ATCC CRL 1597) using 35% polyethylene glycol and cultured in 96-well culture plates. Hybridomas resulting from the fusion were selected in HAT medium. Ten days after the fusion, hybridoma culture supernatants  
25 were screened in an ELISA to test for the presence of monoclonal antibodies binding to the Apo-2 ECD immunoadhesin protein.

In the ELISA, 96-well microtiter plates (Maxisorb; Nunc, Kamstrup, Denmark) were coated by adding 50 µl of 2 µg/ml goat anti-human IgG Fc (purchased from Cappel Laboratories) in PBS to each  
30 well and incubating at 4°C overnight. The plates were then washed three times with wash buffer (PBS containing 0.05% Tween 20). The wells in the microtiter plates were then blocked with 50 µl of 2.0% bovine serum albumin in PBS and incubated at room temperature for 1 hour. The plates were then washed again three times with wash  
35 buffer.

After the washing step, 50 µl of 0.4 µg/ml Apo-2 ECD immunoadhesin protein (as described above) in assay buffer was added to each well. The plates were incubated for 1 hour at room

temperature on a shaker apparatus, followed by washing three times with wash buffer.

Following the wash steps, 100  $\mu$ l of the hybridoma supernatants or purified antibody (using Protein A-sepharose columns) (1  $\mu$ g/ml) was added to designated wells in the presence of CD4-IgG. 100  $\mu$ l of P3X63AgU.1 myeloma cell conditioned medium was added to other designated wells as controls. The plates were incubated at room temperature for 1 hour on a shaker apparatus and then washed three times with wash buffer.

Next, 50  $\mu$ l HRP-conjugated goat anti-mouse IgG Fc (purchased from Cappel Laboratories), diluted 1:1000 in assay buffer (0.5% bovine serum albumin, 0.05% Tween-20, 0.01% Thimersol in PBS), was added to each well and the plates incubated for 1 hour at room temperature on a shaker apparatus. The plates were washed three times with wash buffer, followed by addition of 50  $\mu$ l of substrate (TMB microwell peroxidase substrate, Kirkegaard & Perry, Gaithersburg, MD) to each well and incubation at room temperature for 10 minutes. The reaction was stopped by adding 50  $\mu$ l of TMB 1-component stop solution (diethyl glycol, Kirkegaard & Perry) to each well, and absorbance at 450 nm was read in an automated microtiter plate reader.

Of the hybridoma supernatants screened in the ELISA, 22 supernatants tested positive (calculated as approximately 4 times above background). The supernatants testing positive in the ELISA were further analyzed by FACS analysis using 9D cells (a human B lymphoid cell line expressing Apo-2; Genentech, Inc.) and FITC-conjugated goat anti-mouse IgG. For this analysis, 25  $\mu$ l of cells suspended (at  $4 \times 10^6$  cells/ml) in cell sorter buffer (PBS containing 1% FCS and 0.02%  $\text{NaN}_3$ ) were added to U-bottom microtiter wells, mixed with 100  $\mu$ l of culture supernatant or purified antibody (purified on Protein A-sepharose columns) (10  $\mu$ g /ml) in cell sorter buffer, and incubated for 30 minutes on ice. The cells were then washed and incubated with 100  $\mu$ l FITC-conjugated goat anti-mouse IgG for 30 minutes at 4°C. Cells were then washed twice, resuspended in 150  $\mu$ l of cell sorter buffer and then analyzed by FACScan (Becton Dickinson, Mountain View, CA). FACS analysis showed 8/22 supernatants were positive for anti-Apo-2 antibodies.

Figure 7 shows the FACS staining of 9D cells incubated with one of the Apo-2 antibodies, referred to as 3F11.39.7. As shown in Figure 7, the 3F11.39.7 antibody recognizes the Apo-2 receptor expressed in 9D cells.

5

#### EXAMPLE 10

##### Assay for Ability of Apo-2 Abs to Agonistically induce Apoptosis

10

Hybridoma supernatants and purified antibodies (as described in Example 9 above) were tested for activity to induce Apo-2 mediated 9D cell apoptosis. The 9D cells ( $5 \times 10^5$  cells/0.1ml) were incubated with varying concentrations of antibodies in 100  $\mu$ l complete RPMI media at 4°C for 15 minutes. The cells were then incubated for 5 minutes at 37°C and 10  $\mu$ g of goat anti-mouse IgG Fc antibody (Cappel Laboratories) in 300  $\mu$ l of complete RPMI was added to some of the cell samples. At this point, the cells were incubated overnight at 37°C and in the presence of 7% CO<sub>2</sub>. The cells were then harvested and washed once with PBS. The viability of the cells was determined by staining of FITC-annexin V binding to phosphatidylserine according to manufacturer recommendations (Clontech). The cells were washed in PBS and resuspended in 200  $\mu$ l binding buffer. Ten  $\mu$ l of annexin-V-FITC (1  $\mu$ g/ml) and 10  $\mu$ l of propidium iodide were added to the cells. After incubation for 15 minutes in the dark, the 9D cells were analyzed by FACS.

As shown in Figure 8, the 3F11.39.7 antibody (in the absence of the goat anti-mouse IgG Fc) induced apoptosis in the 9D cells as compared to the control antibodies. Agonistic activity, however, was enhanced by Apo-2 receptor cross-linking in the presence of the goat anti-mouse IgG Fc (see Figure 9). This enhanced apoptosis (Figure 9) by the combination of antibodies is comparable to the apoptotic activity of Apo-2L in 9D cells (data not shown).

EXAMPLE 11Assay for Antibody Ability to Block Apo-2 ligand-induced Apoptosis

5           Hybridoma supernatants and purified antibodies (as described in Example 9 above) were tested for activity to block Apo-2 ligand induced 9D cell apoptosis. The 9D cells ( $5 \times 10^5$  cells/0.1ml) were suspended in complete RPMI media (RPMI plus 10%FCS, glutamine, nonessential amino acids, penicillin, streptomycin, sodium pyruvate) and placed into individual Falcon 2052 tubes. Cells were then incubated with 10  $\mu$ g of antibodies in 200  $\mu$ l media for 15 minutes on ice. 0.2 ml of Apo-2 ligand (2.5  $\mu$ g/ml) (soluble His-tagged Apo-2L prepared as described in WO 97/25428; see also Pitti et al., *supra*) was suspended into complete 15 RPMI media, and then added into the tubes containing the 9D cells. The 9D cells were incubated overnight at 37°C and in the presence of 7% CO<sub>2</sub>. The incubated cells were then harvested and washed once with PBS. The viability of the cells was determined by staining of FITC-annexin V binding to phosphatidylserine according to 20 manufacturer recommendations (Clontech). Specifically, the cells were washed in PBS and resuspended in 200  $\mu$ l binding buffer. Ten  $\mu$ l of annexin-V-FITC (1  $\mu$ g/ml) and 10  $\mu$ l of propidium iodide were added to the cells. After incubation for 15 minutes in the dark, the 9D cells were analyzed by FACS.

25           The results are shown in Figure 10. Since 9D cells express more than one receptor for Apo-2L, Apo-2L can induce apoptosis in the 9D cells by interacting with either Apo-2 or the DR4 receptor. Thus, to detect any blocking activity of the Apo-2 antibodies, the interaction between DR4 and Apo-2L needed to be 30 blocked. In combination with the anti-DR4 antibody, 4H6.17.8 (ATCC HB-12455), the Apo-2 antibody 3F11.39.7 was able to block approximately 50% of apoptosis induced by Apo-2L. The remaining approximately 50% apoptotic activity is believed to be due to the agonistic activities of these two antibodies by themselves, as 35 shown in Figure 10. Accordingly, it is believed that the 3F11.39.7 antibody is a blocking Apo-2 antibody or an antibody which binds Apo-2 in a mode which competes with binding of Apo-2 ligand to Apo-2.

EXAMPLE 12ELISA Assay to Test Binding of Apo-2 Antibodies to Other  
Apo-2 Ligand Receptors

5 An ELISA was conducted to determine if the monoclonal antibody described in Example 9 was able to bind other known Apo-2L receptors beside Apo-2. Specifically, the 3F11.39.7 antibody was tested for binding to DR4 [Pan et al., supra], DcR1 [Sheridan et al., supra], and DcR2 [Marsters et al., Curr. Biol., 7:1003-1006  
10 (1997)]. The ELISA was performed essentially as described in Example 9 above.

The results are shown in Figure 11. The Apo-2 antibody 3F11.39.7 bound to Apo-2. The 3F11.39.7 antibody also showed some cross-reactivity to DR4, but not to DcR1 or DcR2.

15

EXAMPLE 13Antibody Isotyping

The isotype of the 3F11.39.7 antibody (as described above) was determined by coating microtiter plates with isotype  
20 specific goat anti-mouse Ig (Fisher Biotech, Pittsburgh, PA) overnight at 4°C. The plates were then washed with wash buffer (as described in Example 9 above). The wells in the microtiter plates were then blocked with 200 µl of 2% bovine serum albumin (BSA) and incubated at room temperature for one hour. The plates were washed  
25 again three times with wash buffer. Next, 100 µl of 5 µg/ml of purified 3F11.39.7 antibody was added to designated wells. The plates were incubated at room temperature for 30 minutes and then 50 µl HRP-conjugated goat anti-mouse IgG (as described above) was added to each well. The plates were incubated for 30 minutes at  
30 room temperature. The level of HRP bound to the plate was detected using HRP substrate as described above.

The isotyping analysis showed that the 3F11.39.7 antibody is an IgG1 antibody.

EXAMPLE 14Single-Chain Apo-2 AntibodiesA. Antibody Phage Selection using streptavidin-coated paramagnetic beads

5 A phage library was selected using soluble biotinylated antigen and streptavidin-coated paramagnetic beads. The antigen, an Apo-2 ECD immunoadhesin prepared as described in Example 2B above, was biotinylated using IMMUNOPURE NHS-biotin (biotiny-N-hydroxy-succinimide, Pierce) according to manufacturer's  
10 instructions.

Two panning experiments were performed. The first experiment was designed to isolate phage clones specific for Apo-2 and which did not cross react with DR4 or DcR1. Three rounds of panning were carried out. For the first round, 10 µl of the  
15 Cambridge Antibody Technologies phage library were blocked with 1 ml of MPBST (3% dry milk powder, 1X PBS, 0.2% TWEEN) containing 800 µg of CD4-Ig, 300 µg DR4-Ig, and 200 µg of DcR1-Ig for 1 hour on a rotating wheel at room temperature (CD4-Ig, DR4, and DcR1 are described in Capon et al., Nature, 337:525 (1989); Pan et al.,  
20 supra; and Sheridan et al., supra). Biotinylated Apo-2 ECD immunoadhesin was then added to a final concentration of 100 nM, and phage were allowed to bind antigen for 1 hour at 37 °C. Meanwhile, 300 µl of DYNABEADS M-280, coated with streptavidin (DYNAL) were washed 3 times with 1 ml MPBST (using a DYNAL Magnetic  
25 Particle Concentrator) and then blocked for 2 hours at 37 °C with 1 ml fresh MPBST on a rotator. The beads were collected with the MPC, resuspended in 50 µl of MPBST, and added to the phage-plus-antigen solution. Mixing continued on a wheel at room temperature for 15 minutes. The DYNABEADS and attached phage were then washed  
30 a total of 7 times: 3 times with 1 ml PBS-TWEEN, once with MPBS, followed by 3 times with PBS.

Phage were eluted from the beads by incubating 5 minutes at room temperature with 300 µl of 100 mM triethylamine. The phage-containing supernatant was removed and neutralized with 150  
35 µl of 1 M Tris-HCl (pH 7.4). Neutralized phage were used to infect mid-log TG1 host cells and plated on 2YT agar supplemented with 2%

glucose and 100 µg/ml carbenicillin. After overnight growth at 30 °C, colonies were scraped into 10 ml 2YT. 50 µl of this solution was used to inoculate 25 ml of 2YT with carbenicillin and glucose and incubated, shaking, for 2 hours at 37 °C. Helper phage M13K07 (Pharmacia) were added at a m.o.i. of 10. After adsorption, the cells were pelleted and resuspended in 25 ml of 2YT with carbenicillin (100 µg/ml) and kanamycin (50 µg/ml) and growth continued at 30 °C for 4 hours. *E. coli* were removed from the phage by centrifugation, and 1 ml of these phage (approximately 10<sup>12</sup> c.f.u.) were used in subsequent rounds of selection.

For the second round of selection, the 1 ml of harvested phage was adjusted to 3% dry milk, 1X PBS, 0.2% TWEEN and then 100 µg DR4-Ig, 65 µg DcR1-Ig, and 500 µg of CD4-Ig were added for blocking. For selection, biotinylated Apo-2 was added at 10 nM. Washing stringency was increased to two cycles of 7 washes.

For the third round of selection, phage were blocked with only MPBST. Biotinylated Apo-2 was added to 1 nM, and washing stringency was increased to three cycles of 7 washes. Relatively few clones were obtained in this round; therefore Pan 2B, Round 3 was performed using 5 nM of biotinylated Apo-2 with all other conditions repeated as before.

A second panning experiment was performed similarly as above except that in Rounds 1 and 2, blocking of phage solutions was conducted with MPBST containing 1.0 mg/ml CD4-Ig (no other immunoadhesins) and Round 3 was blocked with MPBST only. Biotinylated Apo-2 was added at 200 nM in Round 1, 60 nM in Round 2, and 12 nM in Round 3. At each round, phage were eluted from the magnetic beads with 300 µl of 100 nM triethylamine, then with 300 µl 0.1 M Tris-HCl (pH 7.5), and then with 300 µl glycine-0.1 M HCl (pH 2.2) containing 1 mg/ml BSA. The phage obtained from the three sequential elutions were pooled and used to infect host strain TG1 as above.

#### B. ELISA screening of selected clones

After each round of selection, individual carbenicillin-resistant colonies were screened by ELISA to identify those producing Apo-2-binding phage. Only those clones

which were positive in two or more assay formats were further studied.

Individual clones were inoculated into 2TY with 2% glucose and 100 µg/ml carbenicillin in 96-well tissue culture plates and grown until turbid. Cultures were then infected at a m.o.i. of 10 with M12KO7 helper phage, and infected cells were transferred to 2YT media containing carbenicillin (100 µg/ml) and kanamycin (50 µg/ml) for growth overnight at 30°C with gentle shaking.

NUNC MAXISORP microtiter plates were coated with 50 µl per well of Apo-2 ECD immunoadhesin, or CD4-IgG, at 2 µg/ml in 50 mM carbonate buffer (pH 9.6), at 4°C overnight. After removing antigen, plates were blocked with 3% dry milk in PBS (MPBS) for 2 hours at room temperature.

Phage cultures were centrifuged and 100 µl of phage-containing supernatants were blocked with 20 µl of 6 x PBS / 18% dry milk for 1 hour at room temperature. Block was removed from titer plates and blocked phage added and allowed to bind for 1 hour at room temperature. After washing, phage were detected with a 1:5000 dilution of horseradish peroxidase-conjugated anti-M13 antibody (Pharmacia) in MPBS followed by 3',3',5',5'-tetramethylbenzidine (TMB). Reactions were stopped by the addition of H<sub>2</sub>SO<sub>4</sub> and readings taken by subtracting the A<sub>405nm</sub> from the A<sub>450nm</sub>.

#### C. DNA fingerprinting of clones

The diversity of Apo-2-binding clones was determined by PCR amplifying the scFv insert using primers pUC19R (5'AGC GGA TAA CAA TTT CAC ACA GG 3') (SEQ. ID. NO:12) which anneals upstream of the leader sequence and fdtetseq (5'GTC GTC TTT CCA GAC GGT AGT 3') (SEQ. ID. NO:13) which anneals in the 5' end of gene III, followed by digestion with the frequent-cutting restriction enzyme BstNI.

#### DNA Fingerprinting: Protocol

Mix A:           dH20                           67 µl  
                  10 x ampliTag buffer 10



	25 mM MgCl <sub>2</sub>	10
	DMSO, 50%	2
	forward primer	1
5	Mix B:	
	2.5 mM dNTPs	8 $\mu$ l
	AMPLITAQ	0.5
	reverse primer	1.0

90  $\mu$ l of Mix A was placed in a reaction tube and then inoculated  
 10 with a very small portion of *E. coli* colony using a yellow tip.  
 The reaction mix was then heated in a PCR block to 98°C, for 3  
 minutes, removed, and placed on ice. 10  $\mu$ l Mix B was then added  
 and the reaction mix was thermocycled at 95° C, 30 sec, 55°C 30  
 sec, 72°C 1 minute 20 sec, for 25 cycles in a Perkin Elmer 2400  
 15 thermocycler. 10  $\mu$ l of the resultant reaction product was then  
 removed and run on a 1% agarose gel to test for a 1 kB band. The  
 remaining mix was brought to 1 x BstNI reaction buffer, 5 units  
 BstNI was added and the DNA was allowed to digest for 2 hours at  
 60°C. The resultant samples were then electrophoresed on a  
 20 GeneGel Excel 12.5% acrylamide gel (Pharmacia Biotech).

#### D. Sequencing of clones

The nucleotide sequence of representative clones of  
 each fingerprint pattern were obtained. Colonies were inoculated  
 into 50 ml of LB medium supplemented with 2% glucose and 100  $\mu$ g/ml  
 25 carbenicillin, and grown overnight at 30°C. DNA was isolated  
 using Qiagen Tip-100s and the manufacturer's protocol and cycle  
 sequenced with fluorescent dideoxy chain terminators (Applied  
 Biosystems). Samples were run on an Applied Biosystems 373A  
 Automated DNA Sequencer and sequences analyzed using the program  
 30 "Sequencher" (Gene Codes Corporation). The nucleotides sequences  
 of selected antibodies 16E2, 20E6 and 24C4 are shown in SEQ ID  
 NO:6, SEQ ID NO:7, and SEQ ID NO:8, respectively, (in Figures 15A,  
 15B and 15C respectively). The corresponding amino acid sequences  
 of antibodies 16E2, 20E6 and 24C4 are shown in SEQ ID NO:9, SEQ ID  
 35 NO:10, and SEQ ID NO:11, respectively (and in Figure 16). In  
 addition, Figure 16 identifies the signal region, and heavy and  
 light chain complementarity determining regions (underlined) of  
 these scFv molecules. The CDR regions shown in Figure 16 were

assigned according to the methods of Kabat et al., "Sequences of Proteins of Immunological Interest," NIH Publ. No. 91-3242, 5<sup>th</sup> Edition.

#### E. Purification of scFvs with (his)<sub>6</sub>

5 For protein purification of soluble antibody, *E. coli* strain 33D3 was transformed with phagemid DNA. Five ml of 2YT with carbenicillin and glucose was used to grow overnight cultures at 30°C. 2.5 ml of these cultures were diluted into 250 ml of the same media and grown to an OD<sub>600</sub> of approximately 1.2. The cells  
10 were pelleted and resuspended in 500 ml of 2YT containing IPTG (1 mM) and carbenicillin (100 µg/ml) to induce expression and grown for a further 16 hours at 22°C. Cell pellets were harvested and frozen at -20°C.

The antibodies were purified by immobilized metal  
15 chelate affinity chromatography (IMAC). Frozen pellets were resuspended in 10 ml of ice-cold shockate buffer (25 mM TRIS-HCl, 1 mM EDTA, 500 mM NaCl, 20% sucrose, 1 mM PMSF) by shaking on ice for 1 hour. Imidazole was added to 20 mM, and cell debris removed by centrifugation. The supernatants were adjusted to 1mM MgCl<sub>2</sub>  
20 and 50 mM phosphate buffer pH 7.5. Ni-NTA agarose resin from Qiagen was used according to the manufacturer's instructions. The resin was equilibrated with 50 mM sodium phosphate buffer pH 7.5, 500 mM NaCl, 20 mM imidazole, and the shockate added. Binding occurred in either a batch mode or on a gravity flow column. The  
25 resin was then washed twice with 10 bed volumes of equilibration buffer, and twice with buffer containing imidazole increased to 50mM. Elution of proteins was with 50 mM phosphate buffer pH 7.5, 500 mM NaCl and 250 mM imidazole. Excess salt and imidazole was removed on a PD-10 column (Pharmacia), and proteins were  
30 concentrated using a Centricom 10 to a volume of about 1 ml.

Concentration was estimated spectrophotometrically assuming an A280 nm of 1.0 = 0.6 mg/ml.

#### F. Assays to determine binding specificity of anti-Apo-2 scFvs

35 To evaluate the specificity of each of the scFv clones, ELISA assays were performed to evaluate binding of 16E2, 20E6 and

24C4 to Apo-2 ECD-Ig, DR4-Ig, DcR1-Ig, DcR2-Ig and CD4-Ig (described above and in Example 12).

In brief, NUNC ELISA plates were coated with 50  $\mu$ l of a 1  $\mu$ g/ml receptor-Ig immunoadhesin molecule in 0.05 M sodium carbonate buffer, pH 9.5, and allowed to incubate overnight at 4°C. Plates were then blocked with 285  $\mu$ l ELISA diluent (PBS supplemented with 0.5% BSA, 0.05% Tween 20, pH 7.4) for at least one hour at room temperature. 50  $\mu$ l of the scFvs were added to the plates in a 1:5 serial dilution and allowed to incubate for 1 hour at room temperature. After this 1 hour dilution, the plates were washed 6 times with PBS/0.05% Tween. After binding to antigen coated plates, soluble scFv was detected by adding 50  $\mu$ l of 1  $\mu$ g/ml Mab 9E10 (an anti-c-myc antibody; ATCC CRL 1729) per well and allowing the plates to incubate for 1 hour at room temperature. After washing the plates 6 times with PBS/0.05% Tween, 50  $\mu$ l of a 1:5000 dilution of horseradish peroxidase-conjugated anti-Murine IgG antibody (Cappel catalogue: 55569) in MPBS was added to the plates and allowed to incubate for 1 hour. An observable signal was generated by adding 50  $\mu$ l of 3',3',5',5'-tetramethylbenzidine (TMB) peroxidase substrate (KPL catalogue #: 50-76-00). Reactions were stopped by the addition of H<sub>2</sub>SO<sub>4</sub> and readings taken by subtracting the A<sub>405nm</sub> from the A<sub>450nm</sub>.

As illustrated in Figures 12A, 12B and 12C, the ELISA assays showed that each of these antibodies exhibited a relatively high degree of specificity for Apo-2.

Additional assays utilizing transfected cells also showed the specificity of 16E2 antibody for Apo-2. Specifically, immunohistochemistry experiments were performed to evaluate the binding specificity of the 16E2 antibody to Apo-2 and DR4-transfected CHO cells. CHO cells were transfected with vector alone or vector containing the gene for Apo-2 or DR4. The transfected cells were removed from culture plates, pelleted, and washed twice with PBS. The pellets were then resuspended in O.C.T. (Fisher), flash frozen in isopentain and LN<sub>2</sub>, and later sectioned using standard protocols. Staining of the sectioned cells was performed using a Vectastain Elite ABC kit. The sections were incubated with either anti-Apo-2 antibody 16E2 or a negative control single chain antibody. The secondary antibody

employed was either a biotinylated anti-c-myc 9E10 antibody or anti-Penta His antibody (Qiagen) followed by biotinylated anti-mouse IgG.

This immunohistochemistry assay showed specific staining of the Apo-2-transfected cells but not the DR4-transfected cells. The cellular staining was predominantly cytoplasmic.

#### EXAMPLE 15

##### Assay for Ability of His-tagged scFvs to Agonistically induce Apoptosis

##### A. Annexin V-biotin/Streptavidin-[S-35] 96 Well Assays

Purified scFv antibodies (as described in Example 14 above) were tested for ability to induce Apo-2 mediated apoptosis.

In brief, SK-MES-1 cells (human lung carcinoma cell line; ATCC HTB 58) or HCT 116 cells (human colon carcinoma cell line; ATCC CCL 247) ( $4 \times 10^4$  cells/well) were aliquoted into 96 well plates in assay medium (1:1 mixture of phenol-red free Dulbecco modified Eagle medium and phenol-red free Ham's F-12 nutrient mixture supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 ug/ml streptomycin) and allowed to attach overnight at 37°C. The media was then removed and 0.1 ml of assay medium containing scFv at a final concentration of 50 ug/ml (16E2 or 20E6) was added to the wells (serial dilutions of 1:2 performed in the plates) and allowed to incubate for 1 hour at room temperature. Other single chain antibodies were used as negative controls: an anti-tissue factor scFv clone, 7D5, or a scFv referred to as 19B8. After the 1 hour incubation with scFv antibody, 0.1 ml of 10 ug/ml anti-His (Qiagen, cat. No. 1007671) or anti-c-myc antibodies were added to the appropriate wells. Wells not receiving a crosslinking antibody received media alone. The plates were then allowed to incubate for 30 minutes at room temperature. After the 30 minutes incubation, 0.1 ml of 10 ug/ml goat anti-mouse IgG (ICN cst. No. 67-029) was added to the appropriate wells. Wells not receiving anti-IgG antibody received media alone. The plates were then placed in an incubator for 15 minutes to allow the pH to return to 7.0. For positive controls, a 2 ug/ml solution of Apo-2 ligand (Apo-2L) (prepared as described in Example 11) in potassium phosphate buffer at pH 7.0 was added to the appropriate

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wells, with serial 2 fold dilutions carried out in the plate. The negative control wells received media alone. The cells were then incubated overnight at 37°C in the presence of 5% CO<sub>2</sub>. 0.05 ml of annexin V-biotin (1 ug/ml) in 2X Ca<sup>2+</sup> binding buffer (NeXins B.V.) was then added to the wells and then allowed to mix on a shaker for 30 minutes. 0.05 ml of streptavidin-[S-35] (final concentration of 2.5 x 10<sup>4</sup> cpm/well) (Amersham) in 2X Ca<sup>2+</sup> binding buffer was then added to the wells and then allowed to mix on a shaker for 30 minutes. The plates were then sealed and centrifuged for 4 minutes at 1500 rpm. To assess the extent of apoptosis, the plates were then counted on a TriLux Microbeta Counter (Wallace) to obtain cpm values corresponding to Annexin-V binding.

As shown in Figures 13C and 14B, the 16E2 and 20E6 antibodies agonistically induced apoptosis in SK-MES-1 cells.

#### B. Crystal Violet Assays

In addition to the annexin V-biotin/streptavidin-[S-35] assay described above, scFv antibodies (as described in Example 14 above) were tested for activity to induce Apo-2 mediated apoptosis via assays utilizing crystal violet.

In brief, the SK-MES-1 cells were plated at 4x10<sup>4</sup> cells/well in assay medium (described in Section A above) and allowed to attach overnight at 37°C. The medium was removed and 0.1ml of assay medium containing scFv (as described in Section A above) at a final concentration of 50 µg/ml was added to the appropriate wells (wells without scFv added receive a media change). Selected wells received "pre-complexed" samples in which 10 ug/ml scFv 16E2 was combined with 100 ug/ml anti-His antibody for 5 hours at 4° C with continuous mixing before addition to the plate. The plates were allowed to incubate for 1 hour at room temperature.

The scFv medium was removed and 0.1 ml of 10 µg/ml anti-His (Qiagen, cat. no. 1007671) or anti-c-myc antibodies diluted in assay medium was added to the wells (wells without crosslinker receive a media change.) The plates were then allowed to incubate for 30 minutes at room temperature.

The medium was then removed and 0.1 ml of 10 µg/ml Goat anti-Mouse IgG (Fc Fragment specific-ICN cst. no. 67-029) diluted

in assay medium was added to the appropriate wells (wells without anti-Fc receive a media change). The plates were then placed in the incubator for 15 minutes to allow the pH to return to 7.0.

Apo-2L (stock at 100 µg/ml in potassium phosphate buffer pH 7.0) was diluted to 2 µg/ml and 0.1ml was added to the appropriate wells. Serial two-fold dilutions were carried down the plate. The plates were then incubated overnight at 37°C.

All medium was removed from the wells and the plates were then flooded with crystal violet solution. The plates were allowed to stain for 15 minutes. The crystal violet was removed by flooding the plates with running tap water. The plates were then allowed to dry overnight.

The plates were read on an SLT plate reader at 540nm and the data analyzed using an Excel macro and 4p-fit.

As shown in Figures 13A, 13B, 14A and 14B, the 16E2 and 20E6 antibodies agonistically induced apoptosis in SK-MES-1 cells.

#### EXAMPLE 16

##### Assay for Ability of gD-tagged scFvs to Agonistically Induce Apoptosis

A purified gD-tagged form of 16E2 scFv was tested for ability to induce Apo-2 mediated apoptosis in a crystal violet assay as described in Example 15 above.

##### A. Construction of scFv with gD tag

The Sfi I to Not I fragment of the scFv form of 16E2 was subcloned into a derivative of pAK19 (Carter et al., Methods: A Companion to Methods in Enzymology, 3:183-192 (1991)) containing the phoA promoter and stII signal sequence rather than the lacZ promoter and hybrid signal sequence of the original library. For ease of purification, a DNA fragment coding for 12 amino acids (met-ala-asn-pro-asn-arg-phe-arg-gly-lys-asn-leu SEQ ID NO:14) derived from herpes simplex virus type 1 glycoprotein D (Lasky et al., DNA, 3:23-29 (1984)) was synthesized and inserted at the 3' end of the VL domain in place of the (his)<sub>6</sub> and c-myc epitope originally present in the Cambridge Antibody Technologies library clones.

##### B. Expression in E. coli

The plasmid containing the gene for scFv 16E2-gD was transformed into *E. coli* strain 33D3 for expression in shake flask cultures. 5 ml of 2YT with carbenicillin and glucose was used to grow overnight cultures at 30° C. 2.5 ml of these cultures were diluted into 250 ml of the same medium and grown to an OD<sub>600</sub> of approximately 1.0. The cells were pelleted and resuspended in 500 ml of Modified AP-5 Minimal Media containing carbenicillin (100 µg/ml) and grown for an additional 16 hours at 30° C. The cells were then pelleted and frozen.

10      C. Purification of scFv with gD tag

Frozen cell paste was resuspended at 1gm/10ml of shockate buffer (25 mM Tris-HCl, 1 mM EDTA, 500 mM NaCl, 20% sucrose, 1 mM PMSF, pH 7.2) and gently agitated 4 hours on ice. The cell suspension was then processed through a Polytron microfluidizer (Brinkman). Cell debris was removed by centrifugation at 10,000 x g for 30 minutes. After filtration through a 0.22 micron filter, the supernatant was loaded onto an affinity column (2.5 x 9.0 cm) consisting of an anti-gD antibody 5B6 (Paborsky et al., Protein Engineering, 3:547-553 (1990)) coupled to CNBr Sepharose which had been equilibrated with PBS. The column was washed 18 hours with PBS until the absorbance of the column effluent was equivalent to baseline. All steps were done at 4° C at a linear flow rate of 25 cm/hour. Elution was performed with 0.1 M acetic acid, 0.5 M NaCl, pH 2.9. Column fractions were monitored by absorbance at 280 nm and peak fractions pooled, neutralized with 1.0 M Tris, pH 8.0, dialyzed against PBS and sterile filtered. The resultant protein preparations were analyzed by non-reducing SDS-PAGE.

D. Crystal Violet Assay

30      The apoptosis assay was performed essentially as described in Example 15(B) above except that samples were serially diluted 1:3 in the plates and the 16E2-gD tagged antibody was tested in addition to two other preparations of 16E2 scFv (referred to as Prep. A and Prep. B in Figure 14C). The results of the assay showing apoptosis induction in SK-MES-1 cells by 16E2-gD antibody are illustrated in Figure 14C.

\* \* \* \* \*

Deposit of Material

The following materials have been deposited with the American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia, USA (ATCC):

<u>Material</u>	<u>ATCC Dep. No.</u>	<u>Deposit Date</u>
pRK5-Apo-2	209021	May 8, 1997
3F11.39.7	HB-12456	January 13, 1998

10

This deposit was made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of a viable culture of the deposit for 30 years from the date of deposit. The deposit will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Genentech, Inc. and ATCC, which assures permanent and unrestricted availability of the progeny of the culture of the deposit to the public upon issuance of the pertinent U.S. patent or upon laying open to the public of any U.S. or foreign patent application, whichever comes first, and assures availability of the progeny to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC Section 122 and the Commissioner's rules pursuant thereto (including 37 CFR Section 1.14 with particular reference to 886 OG 638).

The assignee of the present application has agreed that if a culture of the materials on deposit should die or be lost or destroyed when cultivated under suitable conditions, the materials will be promptly replaced on notification with another of the same. Availability of the deposited material is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by the construct deposited, since the deposited embodiment is intended

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as a single illustration of certain aspects of the invention and any constructs that are functionally equivalent are within the scope of this invention. The deposit of material herein does not constitute an admission that the written description herein  
5 contained is inadequate to enable the practice of any aspect of the invention, including the best mode thereof, nor is it to be construed as limiting the scope of the claims to the specific illustrations that it represents. Indeed, various modifications of the invention in addition to those shown and described herein will  
10 become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

5 (i) APPLICANT: Adams, Camilia W.  
Ashkenazi, Avi J.  
Chuntharapai, Anan  
Kim, Kyung J.

10 (ii) TITLE OF INVENTION: Apo-2 Receptor

(iii) NUMBER OF SEQUENCES: 14

15 (iv) CORRESPONDENCE ADDRESS:  
(A) ADDRESSEE: Genentech, Inc.  
(B) STREET: 1 DNA Way  
(C) CITY: South San Francisco  
(D) STATE: California  
(E) COUNTRY: USA  
20 (F) ZIP: 94080

(v) COMPUTER READABLE FORM:  
(A) MEDIUM TYPE: 3.5 inch, 1.44 Mb floppy disk  
(B) COMPUTER: IBM PC compatible  
25 (C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: WinPatin (Genentech)

(vi) CURRENT APPLICATION DATA:  
(A) APPLICATION NUMBER:  
30 (B) FILING DATE:  
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:  
(A) NAME: Marschang, Diane L.  
35 (B) REGISTRATION NUMBER: 35,600  
(C) REFERENCE/DOCKET NUMBER: P1101R2

(ix) TELECOMMUNICATION INFORMATION:  
(A) TELEPHONE: 650/225-5416  
40 (B) TELEFAX: 650/952-9881

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 411 amino acids  
45 (B) TYPE: Amino Acid  
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

50 Met Glu Gln Arg Gly Gln Asn Ala Pro Ala Ala Ser Gly Ala Arg  
1 5 10 15

Lys Arg His Gly Pro Gly Pro Arg Glu Ala Arg Gly Ala Arg Pro  
20 25 30

55 Gly Leu Arg Val Pro Lys Thr Leu Val Leu Val Val Ala Ala Val  
35 40 45

Leu Leu Leu Val Ser Ala Glu Ser Ala Leu Ile Thr Gln Gln Asp  
60 50 55 60

Leu Ala Pro Gln Gln Arg Ala Ala Pro Gln Gln Lys Arg Ser Ser  
65 70 75

	Pro Ser Glu Gly Leu Cys Pro Pro Gly His His Ile Ser Glu Asp	80	85	90
5	Gly Arg Asp Cys Ile Ser Cys Lys Tyr Gly Gln Asp Tyr Ser Thr	95	100	105
	His Trp Asn Asp Leu Leu Phe Cys Leu Arg Cys Thr Arg Cys Asp	110	115	120
10	Ser Gly Glu Val Glu Leu Ser Pro Cys Thr Thr Thr Arg Asn Thr	125	130	135
	Val Cys Gln Cys Glu Glu Gly Thr Phe Arg Glu Glu Asp Ser Pro	140	145	150
15	Glu Met Cys Arg Lys Cys Arg Thr Gly Cys Pro Arg Gly Met Val	155	160	165
	Lys Val Gly Asp Cys Thr Pro Trp Ser Asp Ile Glu Cys Val His	170	175	180
20	Lys Glu Ser Gly Ile Ile Ile Gly Val Thr Val Ala Ala Val Val	185	190	195
	Leu Ile Val Ala Val Phe Val Cys Lys Ser Leu Leu Trp Lys Lys	200	205	210
	Val Leu Pro Tyr Leu Lys Gly Ile Cys Ser Gly Gly Gly Gly Asp	215	220	225
30	Pro Glu Arg Val Asp Arg Ser Ser Gln Arg Pro Gly Ala Glu Asp	230	235	240
	Asn Val Leu Asn Glu Ile Val Ser Ile Leu Gln Pro Thr Gln Val	245	250	255
	Pro Glu Gln Glu Met Glu Val Gln Glu Pro Ala Glu Pro Thr Gly	260	265	270
40	Val Asn Met Leu Ser Pro Gly Glu Ser Glu His Leu Leu Glu Pro	275	280	285
	Ala Glu Ala Glu Arg Ser Gln Arg Arg Arg Leu Leu Val Pro Ala	290	295	300
45	Asn Glu Gly Asp Pro Thr Glu Thr Leu Arg Gln Cys Phe Asp Asp	305	310	315
	Phe Ala Asp Leu Val Pro Phe Asp Ser Trp Glu Pro Leu Met Arg	320	325	330
50	Lys Leu Gly Leu Met Asp Asn Glu Ile Lys Val Ala Lys Ala Glu	335	340	345
	Ala Ala Gly His Arg Asp Thr Leu Tyr Thr Met Leu Ile Lys Trp	350	355	360
	Val Asn Lys Thr Gly Arg Asp Ala Ser Val His Thr Leu Leu Asp	365	370	375
60	Ala Leu Glu Thr Leu Gly Glu Arg Leu Ala Lys Gln Lys Ile Glu	380	385	390
	Asp His Leu Leu Ser Ser Gly Lys Phe Met Tyr Leu Glu Gly Asn			

Ala Asp Ser Ala Xaa Ser

410 411

5

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 1799 base pairs  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

15

CCCACGCGTC CGCATAAATC AGCACGCGGC CGGAGAACCC CGCAATCTCT 50

GCGCCACAA AATACACCGA CGATGCCCGA TCTACTTTAA GGGCTGAAAC 100

20

CCACGGGCCT GAGAGACTAT AAGAGCGTTC CCTACCGCC ATG GAA 145  
Met Glu  
1

25

CAA CGG GGA CAG AAC GCC CCG GCC GCT TCG GGG GCC CGG 184  
Gln Arg Gly Gln Asn Ala Pro Ala Ala Ser Gly Ala Arg  
5 10 15

30

AAA AGG CAC GGC CCA GGA CCC AGG GAG GCG CGG GGA GCC 223  
Lys Arg His Gly Pro Gly Pro Arg Glu Ala Arg Gly Ala  
20 25

35

AGG CCT GGG CTC CGG GTC CCC AAG ACC CTT GTG CTC GTT 262  
Arg Pro Gly Leu Arg Val Pro Lys Thr Leu Val Leu Val  
30 35 40

40

GTC GCC GCG GTC CTG CTG TTG GTC TCA GCT GAG TCT GCT 301  
Val Ala Ala Val Leu Leu Leu Val Ser Ala Glu Ser Ala  
45 50

CTG ATC ACC CAA CAA GAC CTA GCT CCC CAG CAG AGA GCG 340  
Leu Ile Thr Gln Gln Asp Leu Ala Pro Gln Gln Arg Ala  
55 60 65

45

GCC CCA CAA CAA AAG AGG TCC AGC CCC TCA GAG GGA TTG 379  
Ala Pro Gln Gln Lys Arg Ser Ser Pro Ser Glu Gly Leu  
70 75 80

50

TGT CCA CCT GGA CAC CAT ATC TCA GAA GAC GGT AGA GAT 418  
Cys Pro Pro Gly His His Ile Ser Glu Asp Gly Arg Asp  
85 90

55

TGC ATC TCC TGC AAA TAT GGA CAG GAC TAT AGC ACT CAC 457  
Cys Ile Ser Cys Lys Tyr Gly Gln Asp Tyr Ser Thr His  
95 100 105

60

TGG AAT GAC CTC CTT TTC TGC TTG CGC TGC ACC AGG TGT 496  
Trp Asn Asp Leu Leu Phe Cys Leu Arg Cys Thr Arg Cys  
110 115

GAT TCA GGT GAA GTG GAG CTA AGT CCC TGC ACC ACG ACC 535  
Asp Ser Gly Glu Val Glu Leu Ser Pro Cys Thr Thr Thr  
120 125 130

AGA AAC ACA GTG TGT CAG TGC GAA GAA GGC ACC TTC CGG 574  
Arg Asn Thr Val Cys Gln Cys Glu Glu Gly Thr Phe Arg  
135 140 145

5 GAA GAA GAT TCT CCT GAG ATG TGC CGG AAG TGC CGC ACA 613  
Glu Glu Asp Ser Pro Glu Met Cys Arg Lys Cys Arg Thr  
150 155

10 GGG TGT CCC AGA GGG ATG GTC AAG GTC GGT GAT TGT ACA 652  
Gly Cys Pro Arg Gly Met Val Lys Val Gly Asp Cys Thr  
160 165 170

15 CCC TGG AGT GAC ATC GAA TGT GTC CAC AAA GAA TCA GGC 691  
Pro Trp Ser Asp Ile Glu Cys Val His Lys Glu Ser Gly  
175 180

20 ATC ATC ATA GGA GTC ACA GTT GCA GCC GTA GTC TTG ATT 730  
Ile Ile Ile Gly Val Thr Val Ala Ala Val Val Leu Ile  
185 190 195

GTG GCT GTG TTT GTT TGC AAG TCT TTA CTG TGG AAG AAA 769  
Val Ala Val Phe Val Cys Lys Ser Leu Leu Trp Lys Lys  
200 205 210

25 GTC CTT CCT TAC CTG AAA GGC ATC TGC TCA GGT GGT GGT 808  
Val Leu Pro Tyr Leu Lys Gly Ile Cys Ser Gly Gly Gly  
215 220

30 GGG GAC CCT GAG CGT GTG GAC AGA AGC TCA CAA CGA CCT 847  
Gly Asp Pro Glu Arg Val Asp Arg Ser Ser Gln Arg Pro  
225 230 235

35 GGG GCT GAG GAC AAT GTC CTC AAT GAG ATC GTG AGT ATC 886  
Gly Ala Glu Asp Asn Val Leu Asn Glu Ile Val Ser Ile  
240 245

40 TTG CAG CCC ACC CAG GTC CCT GAG CAG GAA ATG GAA GTC 925  
Leu Gln Pro Thr Gln Val Pro Glu Gln Glu Met Glu Val  
250 255 260

CAG GAG CCA GCA GAG CCA ACA GGT GTC AAC ATG TTG TCC 964  
Gln Glu Pro Ala Glu Pro Thr Gly Val Asn Met Leu Ser  
265 270 275

45 CCC GGG GAG TCA GAG CAT CTG CTG GAA CCG GCA GAA GCT 1003  
Pro Gly Glu Ser Glu His Leu Leu Glu Pro Ala Glu Ala  
280 285

50 GAA AGG TCT CAG AGG AGG AGG CTG CTG GTT CCA GCA AAT 1042  
Glu Arg Ser Gln Arg Arg Arg Leu Leu Val Pro Ala Asn  
290 295 300

55 GAA GGT GAT CCC ACT GAG ACT CTG AGA CAG TGC TTC GAT 1081  
Glu Gly Asp Pro Thr Glu Thr Leu Arg Gln Cys Phe Asp  
305 310

GAC TTT GCA GAC TTG GTG CCC TTT GAC TCC TGG GAG CCG 1120  
Asp Phe Ala Asp Leu Val Pro Phe Asp Ser Trp Glu Pro  
315 320 325

60 CTC ATG AGG AAG TTG GGC CTC ATG GAC AAT GAG ATA AAG 1159  
Leu Met Arg Lys Leu Gly Leu Met Asp Asn Glu Ile Lys  
330 335 340

GTG GCT AAA GCT GAG GCA GCG GGC CAC AGG GAC ACC TTG 1198  
 Val Ala Lys Ala Glu Ala Ala Gly His Arg Asp Thr Leu  
 345 350

5 TAC ACG ATG CTG ATA AAG TGG GTC AAC AAA ACC GGG CGA 1237  
 Tyr Thr Met Leu Ile Lys Trp Val Asn Lys Thr Gly Arg  
 355 360 365

10 GAT GCC TCT GTC CAC ACC CTG CTG GAT GCC TTG GAG ACG 1276  
 Asp Ala Ser Val His Thr Leu Leu Asp Ala Leu Glu Thr  
 370 375

15 CTG GGA GAG AGA CTT GCC AAG CAG AAG ATT GAG GAC CAC 1315  
 Leu Gly Glu Arg Leu Ala Lys Gln Lys Ile Glu Asp His  
 380 385 390

20 TTG TTG AGC TCT GGA AAG TTC ATG TAT CTA GAA GGT AAT 1354  
 Leu Leu Ser Ser Gly Lys Phe Met Tyr Leu Glu Gly Asn  
 395 400 405

GCA GAC TCT GCC WTG TCC TAAGTGTG ATTCTCTTCA GGAAGTGAGA 1400  
 Ala Asp Ser Ala Xaa Ser  
 410 411

25 CCTTCCCTGG TTTACCTTTT TTCTGGAAAA AGCCCAACTG GACTCCAGTC 1450

AGTAGGAAAG TGCCACAATT GTCACATGAC CGGTACTGGA AGAAACTCTC 1500

30 CCATCCAACA TCACCCAGTG GATGGAACAT CCTGTAACTT TTCACTGCAC 1550

TTGGCATTAT TTTTATAAGC TGAATGTGAT AATAAGGACA CTATGGAAAT 1600

GTCTGGATCA TTCCGTTTGT GCGTACTTTG AGATTTGGTT TGGGATGTCA 1650

35 TTGTTTTTAC AGCACTTTTT TATCCTAATG TAAATGCTTT ATTTATTIAT 1700

TTGGGCTACA TTGTAAGATC CATCTACAAA AAAAAAAAAA AAAAAAAAAG 1750

40 GCGCGCCGCG ACTCTAGAGT CGACCTGCAG AAGCTTGGCC GCCATGGCC 1799

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

45 (A) LENGTH: 70 base pairs  
 (B) TYPE: Nucleic Acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

50

GGGAGCCGCT CATGAGGAAG TTGGGCCTCA TGGACAATGA GATAAAGGTG 50

GCTAAAGCTG AGGCAGCGGG 70

55

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

60 (A) LENGTH: 29 base pairs  
 (B) TYPE: Nucleic Acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ATCAGGGACT TTCCGCTGGG GACTTTCCG 29

## 5 (2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 30 base pairs  
 (B) TYPE: Nucleic Acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

15

AGGATGGGAA GTGTGTGATA TATCCTTGAT 30

## (2) INFORMATION FOR SEQ ID NO:6:

- 20 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 930 base pairs  
 (B) TYPE: Nucleic Acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGY: Linear

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

30 ATG ACC ATG ATT ACG CCA AGC TTT GGA GCC TTT TTT 36  
 Met Thr Met Ile Thr Pro Ser Phe Gly Ala Phe Phe  
 1 5 10

35 TTG GAG ATT TTC AAC GTG AAA AAA TTA TTA TTC GCA ATT 75  
 Leu Glu Ile Phe Asn Val Lys Lys Leu Leu Phe Ala Ile  
 15 20 25

40 CCT TTA GTT GTT CCT TTC TAT GCG GCC CAG CCG GCC ATG 114  
 Pro Leu Val Val Pro Phe Tyr Ala Ala Gln Pro Ala Met  
 30 35

GCC GAG GTG CAG CTG GTG CAG TCT GGG GGA GGT GTG GAA 153  
 Ala Glu Val Gln Leu Val Gln Ser Gly Gly Gly Val Glu  
 40 45 50

45 CGG CCG GGG GGG TCC CTG AGA CTC TCC TGT GCA GCC TCT 192  
 Arg Pro Gly Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser  
 55 60

50 GGA TTC ACC TTT GAT GAT TAT GGC ATG AGC TGG GTC CGC 231  
 Gly Phe Thr Phe Asp Asp Tyr Gly Met Ser Trp Val Arg  
 65 70 75

55 CAA GCT CCA GGG AAG GGG CTG GAG TGG GTC TCT GGT ATT 270  
 Gln Ala Pro Gly Lys Gly Leu Glu Trp Val Ser Gly Ile  
 80 85 90

AAT TGG AAT GGT GGT AGC ACA GGA TAT GCA GAC TCT GTG 309  
 Asn Trp Asn Gly Gly Ser Thr Gly Tyr Ala Asp Ser Val  
 95 100

60

AAG GGC CGA GTC ACC ATC TCC AGA GAC AAC GCC AAG AAC 348  
 Lys Gly Arg Val Thr Ile Ser Arg Asp Asn Ala Lys Asn  
 105 110 115

TCC CTG TAT CTG CAA ATG AAC AGC' CTG AGA' GCC GAG GAC 387  
 Ser Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp  
 120 125

5 ACG GCC GTA TAT TAC TGT GCG AAA ATC CTG GGT GCC GGA 426  
 Thr Ala Val Tyr Tyr Cys Ala Lys Ile Leu Gly Ala Gly  
 130 135 140

10 CGG GGC TGG TAC TTC GAT CTC TGG GGG AAG GGG ACC ACG 465  
 Arg Gly Trp Tyr Phe Asp Leu Trp Gly Lys Gly Thr Thr  
 145 150 155

15 GTC ACC GTC TCG AGT GGT GGA GGC GGT TCA GGC GGA GGT 504  
 Val Thr Val Ser Ser Gly Gly Gly Gly Ser Gly Gly Gly  
 160 165

20 GGC AGC GGC GGT GGC GGA TCG TCT GAG CTG ACT CAG GAC 543  
 Gly Ser Gly Gly Gly Gly Ser Ser Glu Leu Thr Gln Asp  
 170 175 180

CCT GCT GTG TCT GTG GCC TTG GGA CAG ACA GTC AGG ATC 582  
 Pro Ala Val Ser Val Ala Leu Gly Gln Thr Val Arg Ile  
 185 190

25 ACA TGC CAA GGA GAC AGC CTC AGA AGC TAT TAT GCA AGC 621  
 Thr Cys Gln Gly Asp Ser Leu Arg Ser Tyr Tyr Ala Ser  
 195 200 205

30 TGG TAC CAG CAG AAG CCA GGA CAG GCC CCT GTA CTT GTC 660  
 Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Val Leu Val  
 210 215 220

35 ATC TAT GGT AAA AAC AAC CGG CCC TCA GGG ATC CCA GAC 699  
 Ile Tyr Gly Lys Asn Asn Arg Pro Ser Gly Ile Pro Asp  
 225 230

40 CGA TTC TCT GGC TCC AGC TCA GGA AAC ACA GCT TCC TTG 738  
 Arg Phe Ser Gly Ser Ser Ser Gly Asn Thr Ala Ser Leu  
 235 240 245

ACC ATC ACT GGG GCT CAG GCG GAA GAT GAG GCT GAC TAT 777  
 Thr Ile Thr Gly Ala Gln Ala Glu Asp Glu Ala Asp Tyr  
 250 255

45 TAC TGT AAC TCC CGG GAC AGC AGT GGT AAC CAT GTG GTA 816  
 Tyr Cys Asn Ser Arg Asp Ser Ser Gly Asn His Val Val  
 260 265 270

50 TTC GGC GGA GGG ACC AAG CTG ACC GTC CTA GGT GCG GCC 855  
 Phe Gly Gly Gly Thr Lys Leu Thr Val Leu Gly Ala Ala  
 275 280 285

55 GCA CAT CAT CAT CAC CAT CAC GGG GCC GCA GAA CAA AAA 894  
 Ala His His His His His His Gly Ala Ala Glu Gln Lys  
 290 295

60 CTC ATC TCA GAA GAG GAT CTG AAT GGG GCC GCA TAG 930  
 Leu Ile Ser Glu Glu Asp Leu Asn Gly Ala Ala  
 300 305 309

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 939 base pairs



(B) TYPE: Nucleic Acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGY: Linear

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATG ACC ATG ATT ACG CCA AGC TTT GGA GCC TTT TTT 36  
 Met Thr Met Ile Thr Pro Ser Phe Gly Ala Phe Phe  
 10 1 5 10

TTG GAG ATT TTC AAC GTG AAA AAA TTA TTA TTC GCA ATT 75  
 Leu Glu Ile Phe Asn Val Lys Lys Leu Leu Phe Ala Ile  
 15 15 20 25

CCT TTA GTT GTT CCT TTC TAT GCG GCC CAG CCG GCC ATG 114  
 Pro Leu Val Val Pro Phe Tyr Ala Ala Gln Pro Ala Met  
 30 35

GCC GGG GTG CAG CTG GTG GAG TCT GGG GGA GGC TTG GTC 153  
 Ala Gly Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val  
 40 45 50

CAG CCT GGG GGG TCC CTG AGA CTC TCC TGT GCA GCC TCT 192  
 Gln Pro Gly Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser  
 25 55 60

GGA TTC ACC TTT AGT AGC TAT TGG ATG AGC TGG GTC CGC 231  
 Gly Phe Thr Phe Ser Ser Tyr Trp Met Ser Trp Val Arg  
 30 65 70 75

CAG GCT CCA GGG AAG GGG CTG GAG TGG GTG GCC AAC ATA 270  
 Gln Ala Pro Gly Lys Gly Leu Glu Trp Val Ala Asn Ile  
 80 85 90

AAG CAA GAT GGA AGT GAG AAA TAC TAT GTG GAC TCT GTG 309  
 Lys Gln Asp Gly Ser Glu Lys Tyr Tyr Val Asp Ser Val  
 35 95 100

AAG GGC CGA TTC ACC ATC TCC AGA GAC AAC GCC AAG AAC 348  
 Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn  
 40 105 110 115

TCA CTG TAT CTG CAA ATG AAC AGC CTG AGA GCC GAG GAC 387  
 Ser Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp  
 45 120 125

ACG GCT GTG TAT TAC TGT GCG AGA GAT CTT TTA AAG GTC 426  
 Thr Ala Val Tyr Tyr Cys Ala Arg Asp Leu Leu Lys Val  
 50 130 135 140

AAG GGC AGC TCG TCT GGG TGG TTC GAC CCC TGG GGG AGA 465  
 Lys Gly Ser Ser Ser Gly Trp Phe Asp Pro Trp Gly Arg  
 145 150 155

GGG ACC ACG GTC ACC GTC TCG AGT GGT GGA GGC GGT TCA 504  
 Gly Thr Thr Val Thr Val Ser Ser Gly Gly Gly Gly Ser  
 55 160 165

GGC GGA GGT GGT AGC GGC GGT GGC GGA TCG TCT GAG CTG 543  
 Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Ser Glu Leu  
 60 170 175 180

ACT CAG GAC CCT GCT GTG TCT GTG GCC TTG GGA CAG ACA 582

Thr Gln Asp Pro Ala Val Ser Val Ala Leu Gly Gln Thr  
185 190

5 GTC AGG ATC ACA TGC CAA GGA GAC AGC CTC AGA AGC TAT 621  
Val Arg Ile Thr Cys Gln Gly Asp Ser Leu Arg Ser Tyr  
195 200 205

10 TAT GCA AGC TGG TAC CAG CAG AAG CCA GGA CAG GCC CCT 660  
Tyr Ala Ser Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro  
210 215 220

15 GTA CTT GTC ATC TAT GGT AAA AAC AAC CGG CCC TCA GGG 699  
Val Leu Val Ile Thr Gly Lys Asn Asn Arg Pro Ser Gly  
225 230

ATC CCA GAC CGA TTC TCT GGC TCC AGC TCA GGA AAC ACA 738  
Ile Pro Asp Arg Phe Ser Gly Ser Ser Ser Gly Asn Thr  
235 240 245

20 GCT TCC TTG ACC ATC ACT GGG GCT CAG GCG GAA GAT GAG 777  
Ala Ser Leu Thr Ile Thr Gly Ala Gln Ala Glu Asp Glu  
250 255

25 GCT GAC TAT TAC TGT AAC TCC CGG GAC AGC AGT GGT AAC 816  
Ala Asp Tyr Tyr Cys Asn Ser Arg Asp Ser Ser Gly Asn  
260 265 270

30 CAT GTG GTA TTC GGC GGA GGG ACC AAG CTG ACC GTC CTA 855  
His Val Val Phe Gly Gly Gly Thr Lys Leu Thr Val Leu  
275 280 285

35 GGT GCG GCC GCA CAT CAT CAT CAC CAT CAC GGG GCC GCA 894  
Gly Ala Ala Ala His His His His His His Gly Ala Ala  
290 295

GAA CAA AAA CTC ATC TCA GAA GAG GAT CTG AAT GGG GCC 933  
Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu Asn Gly Ala  
300 305 310

40 GCA TAG 939  
Ala  
312

## (2) INFORMATION FOR SEQ ID NO:8:

- 45 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 933 base pairs  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
50 (D) TOPOLOGY: Linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

55 ATG ACC ATG ATT ACG CCA AGC TTT GGA GCC TTT TTT 36  
Met Thr Met Ile Thr Pro Ser Phe Gly Ala Phe Phe  
1 5 10

60 TTG GAG ATT TTC AAC GTG AAA AAA TTA TTA TTC GCA ATT 75  
Leu Glu Ile Phe Asn Val Lys Lys Leu Leu Phe Ala Ile  
15 20 25

CCT TTA GTT GTT CCT TTC TAT GCG GCC CAG CCG GCC ATG 114  
Pro Leu Val Val Pro Phe Tyr Ala Ala Gln Pro Ala Met

5 GCC CAG GTG CAG CTG GTG CAG TCT GGG GGA GGC GTG GTC 153  
 Ala Gln Val Gln Leu Val Gln Ser Gly Gly Gly Val Val  
 40 45 50

10 CAG CCT GGG CGG TCC CTG AGA CTC TCC TGT GCA GCT TCT 192  
 Gln Pro Gly Arg Ser Leu Arg Leu Ser Cys Ala Ala Ser  
 55 60

15 GGG TTC ATT TTC AGT AGT TAT GGG ATG CAC TGG GTC CGC 231  
 Gly Phe Ile Phe Ser Ser Tyr Gly Met His Trp Val Arg  
 65 70 75

20 CAG GCT CCA GGC AAG GGG CTG GAG TGG GTG GCA GGT ATT 270  
 Gln Ala Pro Gly Lys Gly Leu Glu Trp Val Ala Gly Ile  
 80 85 90

25 TTT TAT GAT GGA GGT AAT AAA TAC TAT GCA GAC TCC GTG 309  
 Phe Tyr Asp Gly Gly Asn Lys Tyr Tyr Ala Asp Ser Val  
 95 100

30 AAG GGC CGA TTC ACC ATC TCC AGA GAC AAT TCC AAG AAC 348  
 Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn  
 105 110 115

35 ACG CTG TAT CTG CAA ATG AAC AGC CTG AGA GCT GAG GAC 387  
 Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp  
 120 125

40 ACG GCT GTG TAT TAC TGT GCG AGA GAT AGG GGC TAC TAC 426  
 Thr Ala Val Tyr Tyr Cys Ala Arg Asp Arg Gly Tyr Tyr  
 130 135 140

45 TAC ATG GAC GTC TGG GGC AAA GGG ACC ACG GTC ACC GTC 465  
 Tyr Met Asp Val Trp Gly Lys Gly Thr Thr Val Thr Val  
 145 150 155

50 TCC TCA GGT GGA GGC GGT TCA GGC GGA GGT GGC TCT GGC 504  
 Ser Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly  
 160 165

55 GGT GGC GGA TCG CAG TCT GTG TTG ACG CAG CCG CCC TCA 543  
 Gly Gly Gly Ser Gln Ser Val Leu Thr Gln Pro Pro Ser  
 170 175 180

60 GTG TCT GGG GCC CCA GGA CAG AGG GTC ACC ATC TCC TGC 582  
 Val Ser Gly Ala Pro Gly Gln Arg Val Thr Ile Ser Cys  
 185 190

65 ACT GGG AGA AGC TCC AAC ATC GGG GCA GGT CAT GAT GTA 621  
 Thr Gly Arg Ser Ser Asn Ile Gly Ala Gly His Asp Val  
 195 200 205

70 CAC TGG TAC CAG CAA CTT CCA GGA ACA GCC CCC AAA CTC 660  
 His Trp Tyr Gln Gln Leu Pro Gly Thr Ala Pro Lys Leu  
 210 215 220

75 CTC ATC TAT GAT GAC AGC AAT CGG CCC TCA GGG GTC CCT 699  
 Leu Ile Tyr Asp Asp Ser Asn Arg Pro Ser Gly Val Pro  
 225 230

80 GAC CGA TTC TCT GGC TCC AGG TCT GGC ACC TCA GCC TCC 738  
 Asp Arg Phe Ser Gly Ser Arg Ser Gly Thr Ser Ala Ser

235 240 245

CTG GCC ATC ACT GGG CTC CAG GCT GAA GAT GAG GCT GAT 777  
 Leu Ala Ile Thr Gly Leu Gln Ala Glu Asp Glu Ala Asp  
 5 250 255

TAT TAC TGC CAG TCC TAT GAC AGC AGC CTG AGG GGT TCG 816  
 Tyr Tyr Cys Gln Ser Tyr Asp Ser Ser Leu Arg Gly Ser  
 10 260 265 270

GTA TTC GGC GGA GGG ACC AAG GTC ACT GTC CTA GGT GCG 855  
 Val Phe Gly Gly Gly Thr Lys Val Thr Val Leu Gly Ala  
 275 280 285

GCC GCA CAT CAT CAT CAC CAT CAC GGG GCC GCA GAA CAA 894  
 Ala Ala His His His His His His Gly Ala Ala Glu Gln  
 15 290 295

AAA CTC ATC TCA GAA GAG GAT CTG AAT GGG GCC GCA 930  
 Lys Leu Ile Ser Glu Glu Asp Leu Asn Gly Ala Ala  
 20 300 305 310

TAG 933

25 (2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 309 amino acids  
 (B) TYPE: Amino Acid  
 (D) TOPOLOGY: Linear  
 30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Met Thr Met Ile Thr Pro Ser Phe Gly Ala Phe Phe Leu Glu Ile  
 35 1 5 10 15

Phe Asn Val Lys Lys Leu Leu Phe Ala Ile Pro Leu Val Val Pro  
 20 25 30

Phe Tyr Ala Ala Gln Pro Ala Met Ala Glu Val Gln Leu Val Gln  
 40 35 40 45

Ser Gly Gly Gly Val Glu Arg Pro Gly Gly Ser Leu Arg Leu Ser  
 50 55 60

Cys Ala Ala Ser Gly Phe Thr Phe Asp Asp Tyr Gly Met Ser Trp  
 45 65 70 75

Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val Ser Gly Ile  
 50 80 85 90

Asn Trp Asn Gly Gly Ser Thr Gly Tyr Ala Asp Ser Val Lys Gly  
 95 100 105

Arg Val Thr Ile Ser Arg Asp Asn Ala Lys Asn Ser Leu Tyr Leu  
 55 110 115 120

Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys  
 125 130 135

Ala Lys Ile Leu Gly Ala Gly Arg Gly Trp Tyr Phe Asp Leu Trp  
 60 140 145 150

Gly Lys Gly Thr Thr Val Thr Val Ser Ser Gly Gly Gly Gly Ser

155 160 165

Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Ser Glu Leu Thr Gln  
170 175 180

5 Asp Pro Ala Val Ser Val Ala Leu Gly Gln Thr Val Arg Ile Thr  
185 190 195

10 Cys Gln Gly Asp Ser Leu Arg Ser Tyr Tyr Ala Ser Trp Tyr Gln  
200 205 210

Gln Lys Pro Gly Gln Ala Pro Val Leu Val Ile Tyr Gly Lys Asn  
215 220 225

15 Asn Arg Pro Ser Gly Ile Pro Asp Arg Phe Ser Gly Ser Ser Ser  
230 235 240

Gly Asn Thr Ala Ser Leu Thr Ile Thr Gly Ala Gln Ala Glu Asp  
245 250 255

20 Glu Ala Asp Tyr Tyr Cys Asn Ser Arg Asp Ser Ser Gly Asn His  
260 265 270

25 Val Val Phe Gly Gly Gly Thr Lys Leu Thr Val Leu Gly Ala Ala  
275 280 285

Ala His His His His His His Gly Ala Ala Glu Gln Lys Leu Ile  
290 295 300

30 Ser Glu Glu Asp Leu Asn Gly Ala Ala  
305 309

## (2) INFORMATION FOR SEQ ID NO:10:

35 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 312 amino acids  
(B) TYPE: Amino Acid  
(D) TOPOLOGY: Linear

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Met Thr Met Ile Thr Pro Ser Phe Gly Ala Phe Phe Leu Glu Ile  
1 5 10 15

45 Phe Asn Val Lys Lys Leu Leu Phe Ala Ile Pro Leu Val Val Pro  
20 25 30

Phe Tyr Ala Ala Gln Pro Ala Met Ala Gly Val Gln Leu Val Glu  
35 40 45

50 Ser Gly Gly Gly Leu Val Gln Pro Gly Gly Ser Leu Arg Leu Ser  
50 55 60

55 Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser Tyr Trp Met Ser Trp  
65 70 75

Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val Ala Asn Ile  
80 85 90

60 Lys Gln Asp Gly Ser Glu Lys Tyr Tyr Val Asp Ser Val Lys Gly  
95 100 105

Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Ser Leu Tyr Leu  
110 115 120

Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys  
 125 130 135  
 5 Ala Arg Asp Leu Leu Lys Val Lys Gly Ser Ser Ser Gly Trp Phe  
 140 145 150  
 Asp Pro Trp Gly Arg Gly Thr Thr Val Thr Val Ser Ser Gly Gly  
 155 160 165  
 10 Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Ser Glu  
 170 175 180  
 Leu Thr Gln Asp Pro Ala Val Ser Val Ala Leu Gly Gln Thr Val  
 185 190 195  
 Arg Ile Thr Cys Gln Gly Asp Ser Leu Arg Ser Tyr Tyr Ala Ser  
 200 205 210  
 20 Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Val Leu Val Ile Tyr  
 215 220 225  
 Gly Lys Asn Asn Arg Pro Ser Gly Ile Pro Asp Arg Phe Ser Gly  
 230 235 240  
 25 Ser Ser Ser Gly Asn Thr Ala Ser Leu Thr Ile Thr Gly Ala Gln  
 245 250 255  
 Ala Glu Asp Glu Ala Asp Tyr Tyr Cys Asn Ser Arg Asp Ser Ser  
 260 265 270  
 Gly Asn His Val Val Phe Gly Gly Gly Thr Lys Leu Thr Val Leu  
 275 280 285  
 35 Gly Ala Ala Ala His His His His His His Gly Ala Ala Glu Gln  
 290 295 300  
 Lys Leu Ile Ser Glu Glu Asp Leu Asn Gly Ala Ala  
 305 310 312  
 40

## (2) INFORMATION FOR SEQ ID NO:11:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 310 amino acids  
 (B) TYPE: Amino Acid  
 (D) TOPOLOGY: Linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

50 Met Thr Met Ile Thr Pro Ser Phe Gly Ala Phe Phe Leu Glu Ile  
 1 5 10 15  
 Phe Asn Val Lys Lys Leu Leu Phe Ala Ile Pro Leu Val Val Pro  
 20 25 30  
 55 Phe Tyr Ala Ala Gln Pro Ala Met Ala Gln Val Gln Leu Val Gln  
 35 40 45  
 Ser Gly Gly Gly Val Val Gln Pro Gly Arg Ser Leu Arg Leu Ser  
 50 55 60  
 Cys Ala Ala Ser Gly Phe Ile Phe Ser Ser Tyr Gly Met His Trp  
 65 70 75

	Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val Ala Gly Ile	80	85	90
5	Phe Tyr Asp Gly Gly Asn Lys Tyr Tyr Ala Asp Ser Val Lys Gly	95	100	105
	Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr Leu	110	115	120
10	Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys	125	130	135
	Ala Arg Asp Arg Gly Tyr Tyr Tyr Met Asp Val Trp Gly Lys Gly	140	145	150
15	Thr Thr Val Thr Val Ser Ser Gly Gly Gly Gly Ser Gly Gly Gly	155	160	165
	Gly Ser Gly Gly Gly Gly Ser Gln Ser Val Leu Thr Gln Pro Pro	170	175	180
20	Ser Val Ser Gly Ala Pro Gly Gln Arg Val Thr Ile Ser Cys Thr	185	190	195
	Gly Arg Ser Ser Asn Ile Gly Ala Gly His Asp Val His Trp Tyr	200	205	210
	Gln Gln Leu Pro Gly Thr Ala Pro Lys Leu Leu Ile Tyr Asp Asp	215	220	225
30	Ser Asn Arg Pro Ser Gly Val Pro Asp Arg Phe Ser Gly Ser Arg	230	235	240
	Ser Gly Thr Ser Ala Ser Leu Ala Ile Thr Gly Leu Gln Ala Glu	245	250	255
35	Asp Glu Ala Asp Tyr Tyr Cys Gln Ser Tyr Asp Ser Ser Leu Arg	260	265	270
	Gly Ser Val Phe Gly Gly Gly Thr Lys Val Thr Val Leu Gly Ala	275	280	285
	Ala Ala His His His His His His Gly Ala Ala Glu Gln Lys Leu	290	295	300
45	Ile Ser Glu Glu Asp Leu Asn Gly Ala Ala	305	310	

(2) INFORMATION FOR SEQ ID NO:12:

- 50 (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 23 base pairs
  - (B) TYPE: Nucleic Acid
  - (C) STRANDEDNESS: Single
  - 55 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

60 AGCGGATAAC AATTCACAC AGG 23

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GTCGTCTTTC CAGAGGGTAG T 21

10

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 amino acids
- (B) TYPE: Amino Acid
- (D) TOPOLOGY: Linear

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

20 Met Ala Asp Pro Asn Arg Phe Arg Gly Lys Asp Leu  
 1 5 10 12

10052798-11001



## What is claimed is:

1. Isolated Apo-2 polypeptide having at least 80% amino acid sequence identity with native sequence Apo-2 polypeptide comprising amino acid residues 1 to 411 of SEQ ID NO:1.
- 5 2. The Apo-2 polypeptide of claim 1 wherein said polypeptide has at least 90% amino acid sequence identity.
3. The Apo-2 polypeptide of claim 2 wherein said polypeptide has at least 95% amino acid sequence identity.
4. Isolated Apo-2 polypeptide comprising amino acid residues 1 to  
10 411 of SEQ ID NO:1.
5. Isolated extracellular domain sequence of Apo-2 polypeptide comprising amino acid residues 54 to 182 of SEQ ID NO:1.
6. The extracellular domain sequence of claim 5 comprising amino acid residues 1 to 182 of SEQ ID NO:1.
- 15 7. Isolated death domain sequence of Apo-2 polypeptide comprising amino acid residues 324 to 391 of SEQ ID NO:1.
8. A chimeric molecule comprising the Apo-2 polypeptide of claim 1 or the extracellular domain sequence of claim 5 fused to a heterologous amino acid sequence.
- 20 9. The chimeric molecule of claim 8 wherein said heterologous amino acid sequence is an epitope tag sequence.
10. The chimeric molecule of claim 8 wherein said heterologous amino acid sequence is an immunoglobulin sequence.
11. The chimeric molecule of claim 10 wherein said immunoglobulin  
25 sequence is an IgG.
12. Isolated nucleic acid comprising a DNA encoding the polypeptide of claim 1, the extracellular domain sequence of claim 5, or the death domain sequence of claim 7.
13. The nucleic acid of claim 12 wherein said DNA encodes an Apo-2  
30 polypeptide comprising amino acid residues 1 to 411 of SEQ ID NO:1.
14. A vector comprising the nucleic acid of claim 12.
15. The vector of claim 14 operably linked to control sequences recognized by a host cell transformed with the vector.
- 35 16. The vector of claim 14 comprising ATCC deposit accession number 209021.
17. A host cell comprising the vector of claim 14.
18. The host cell of claim 17 comprising a CHO cell.
19. The host cell of claim 17 comprising *E. coli*.

20. The host cell of claim 17 comprising a yeast cell.
21. A process of producing an Apo-2 polypeptide comprising culturing the host cell of claim 17 under conditions sufficient to express Apo-2 polypeptide and recovering the expressed Apo-2 polypeptide from the culture.
22. An Apo-2 polypeptide which is obtained or obtainable by expressing the polypeptide encoded by the cDNA insert in ATCC deposit accession number 209021.
23. A non-human, transgenic animal which contains cells that express DNA encoding Apo-2 polypeptide.
24. The animal of claim 23 which is a mouse or rat.
25. A non-human, knockout animal which contains cells having an altered gene encoding Apo-2 polypeptide.
26. The animal of claim 25 which is a mouse or rat.
27. An antibody which specifically binds to an Apo-2 polypeptide.
28. The antibody of claim 27 which is a monoclonal antibody.
29. The antibody of claim 27 comprising an agonistic antibody.
30. The antibody of claim 27 comprising a blocking antibody.
31. The antibody of claim 24 comprising a chimeric antibody.
32. The antibody of claim 28 wherein said antibody is an IgG antibody.
33. The antibody of claim 28 wherein said antibody comprises an Fab fragment.
34. The antibody of claim 28 wherein said antibody comprises a scFv fragment.
35. The antibody of claim 28 wherein said antibody comprises a F(ab')<sub>2</sub> fragment.
36. The antibody of claim 27 wherein said antibody comprises a human antibody.
37. The antibody of claim 28 having the biological characteristics of the monoclonal antibody produced by the hybridoma cell line deposited as ATCC accession number HB-12456.
38. The antibody of claim 28 wherein the antibody binds to the same epitope as the epitope to which the monoclonal antibody produced by the hybridoma cell line deposited as ATCC accession number HB-12456 binds.
39. A hybridoma cell line which produces the antibody of claim 28.
40. The hybridoma cell line deposited as ATCC accession number

HB-12456.

41. The monoclonal antibody produced by the hybridoma cell line deposited as ATCC accession number HB-12456.
42. The antibody of claim 27 wherein said antibody is a single-chain antibody.
43. The antibody of claim 42 wherein said antibody comprises the 16E2 antibody.
44. The antibody of claim 42 wherein said antibody comprises the 20E6 antibody.
45. The antibody of claim 42 wherein said antibody comprises the 24C4 antibody.
46. The antibody of claim 42 wherein said antibody is fused to an epitope tag sequence.
47. A chimeric molecule comprising the antibody of claim 27 fused to a heterologous amino acid sequence.
48. The chimeric molecule of claim 47 wherein said heterologous amino acid sequence comprises an immunoglobulin sequence.
49. A dimeric molecule comprising the Apo-2 antibody of claim 27 and a heterologous antibody.
50. A homodimeric molecule comprising a first Apo-2 antibody and a second Apo-2 antibody.
51. Isolated nucleic acid comprising DNA encoding the Apo-2 antibody of claim 43.
52. Isolated nucleic acid comprising DNA encoding the antibody of claim 44.
53. Isolated nucleic acid comprising DNA encoding the antibody of claim 45.
54. A vector comprising the nucleic acid of claim 51, 52, or 53.
55. A host cell comprising the vector of claim 54.
56. A method of producing an Apo-2 antibody comprising culturing the host cell of claim 55 under conditions wherein the DNA is expressed.
57. A composition comprising the antibody of claim 27 and a carrier.
58. The composition of claim 57 wherein said carrier is a pharmaceutically-acceptable carrier.
59. A method of inducing apoptosis in mammalian cancer cells comprising exposing mammalian cancer cells to an effective amount of the Apo-2 agonistic antibody of claim 29.

60. The method of claim 59 wherein said agonistic antibody comprises a single-chain antibody.
61. A method of treating mammalian cancer cells comprising exposing mammalian cancer cells to an agent which activates Apo-2.
- 5 62. The method of claim 61 wherein said agent comprises an agonistic Apo-2 antibody.
63. An article of manufacture comprising a container and a composition contained within said container, wherein the composition includes Apo-2 polypeptide or Apo-2 antibody.
- 10 64. The article of manufacture of claim 63 further comprising instructions for using the Apo-2 polypeptide or Apo-2 antibody in vivo or ex vivo.

10052798.110204

Abstract of the Disclosure

Novel polypeptides, designated Apo-2, which are capable of modulating apoptosis are provided. Compositions including Apo-2 chimeras, nucleic acid encoding Apo-2, and antibodies to Apo-2 are  
 5 also provided.

10052798 110201

1 CCCACGGGTC CGCATAAATC AGCAGCGGGC CGGAGAAGCC CGCAATCTCT GCGCCACAAA AATACACCGA CGATGCCCGA TCTACTTTAA GGGCTGAAC  
GGGTGGCAG CCGTATTTAG TCGTGGCGC GCTCTTGG CCGGTAGAGA CCGGGTCTT TTATGTGGCT GCTACGGGCT AGATGAAATT CCGGACTTTG

101 CCACGGGGCT GAGAGACTAT AAGAGCGTTC CCTACCGCCA TGAACAACG GGGACAGAAC GCGCGGGCGG CTTCCGGGGC CCGGAAAAGG CACGGGGCCAG  
GTCGCCGGA CTCTGTGATA TTCTCGCAAG GGATGGCGGT ACCTGTGTC CCGTGTCTTG CCGGGCGGGC GAAGCCCCG GGCCTTTTC GTGCCGGGTC  
1 M etGluglnar gGlyGlnasn AlaProAlaA laserGlyAl aArgLysArg HisGlyProGly

201 GACCCAGGGA GCGCGGGGA GCCAGGGCTG GGTCCGGGT CCCCAGACC CTTGTGCTCG TTGTCCCGC GGTCTCTG TTGGTCTCAG CTGAGTCTGC  
CTGGTCCCT CCGGCCCT CCGTCCGAC CGTCCGGC CGAGGCCCA GGGTCTTGG GAACACGAGC AACAGCGGG CCAGGACGAC AACCAGAGTC GACTCAGACG

22 ProArgG1 uAlaArgGly AlaArgProG lyLeuArgVa lProLysThr LeuValLeuV alValAlaAl aValLeuLeu LeuValserA laGluSerAla

301 TCTGATCACC CAACAGACC TAGTCCCCA GACAGAGCG GCCCCCAAC AAAAGAGGTC CAGCCCTCA GAGGGATTGT GTCCACCTGG ACACCATATC  
AGACTAGTGG GTTGTCTCG ATCGAGGGGT CGTCTCTCG CGGGGTGTG TTTTCTCCAG GTCCGGGAGT CTCCTTAACA CAGGTGGACC TGTGGTATAG

55 LeuileThr GlnGlnAspL euAlaProG1 nGlnArgAla AlaProGlnG lnLysArgse rserProser GluGlyLeuC ysProProG1 yHisHisIle

401 TCAGAAAGAC GTAGAGATTG CATCTCCTG CAATATGAC AGGACTATAG CACTCACTGG AATGACCTCC TTTTCTGCTT GCGTGCACC AGGTGTGATT  
AGTCTTCTGC CATCTTAAC GTAGAGGACG TTTATACCTG TCCTGATATC GTGAGTGACC TTACTGGAGG AAAAGACGAA GCGCAGCTGG TCCACACTAA

-88 serGluasp lArgaspCy sileserCys LysTyrglyG lnAspTyrs e rThrHisTrp AsnAspLeuL eupheCysLe uArgCysThr ArgCysaspser

501 CAGGTGAAGT GGAGCTAAGT CCCTGCACCA CGACAGAAA CACAGTGTGT CAGTGCAGAG AAGCACCTT CCGGGAAGAA GATTCTCCTG AGATGTGCCG  
GTCCACTTCA CTTCAATTCA GGGACGTGT GGTGTCTTT GTGTACACA GTACAGCTTC TTCCGTGAA GCGCTTGGAA GCGCTTCTT CTAAGAGGAC TCTACACGGC

122 GlyGluVa lGluLeuser ProCysThrT hrThrArgas nThrValCys GlnCysGluG luclyThrPh eArgGluGlu AspserProG lumetCysArg

601 GAAGTCCCG ACAGGTCTC CCAGAGGGAT GGTCAAGGTC GGTGATTGTA CACCTCGAG TGACATCGAA TGTGTCCACA AAGAATCAGG CATCATCATA  
CTTCACGGCG TGTCCACAG GGTCTCCCTA CCAGTCCAG CCCTAACAT GTGGGACCTC ACTGTAGCTT ACACAGGTGT TTCTTAGTCC GTAGTAGTAT

155 LysCysArg ThrGlyCysP roArgGlyMe tValLysVal tValLysVal GlyAspCysT hrProTrpse rAspIleGlu CysValHisL ysGluSerG1 yIleIleIle

701 GGAGTCACAG TTGACGCCGT AGTCTTGATT GTGGCTGTGT TTGTTTGCAA GTCTTTACTG TCGAAGAAAG TCCTTCTCTTA CCGTGAAGGC ATCTGCTCAG  
CCTCAGTGT AACGTGGCA TCAGAACTAA CACCGACACA CACAAACGTT CAGAAATGAC ACCTTCTTC AGGAAGGAAT GGACTTTCCG TAGACGAGTC

188 GlyValThrV alAlaAlaVa lValLeuile heValCysLy sserLeuLeu TrpLysLysv alLeuProTy rLeuLysGly IleCysSerGly

801 GTGGTGGTGG GGACCTGAG CGTGTGACA GAAGCTCACA ACGACCTGG GCTGAGGACA ATGTCTCAA TGAGATCGTG AGTATCTTGC AGCCACCCCA  
CACCAACACC CCTGGGACTC CCACACCTGT CTTCCGAGTGT TGTGGACCC CGACTCCTGT TACAGGAGTT ACTCTAGCAC TCATAGAACG TCGGGTGGGT

222 GlyGlyG1 yAspProGlu ArgValAspa rgSerSerG1 nArgProGly AlaGluAspa snValLeuAs nGluileVal SerIleLeuG lnProThrGln

901 GGTCCCTGAG CAGGAAATGG AAGTCCAGGA GCCAGCAGAG CCAACAGGTG TCAACATGTT GTCCCGCGG GAGTCAGAGC ATCTGCTGGA ACCGGCAGAA  
CCAGGGACTC GTCCTTTACC TTCAGGTCTT CCGTCTGCTC GGTGTCCAC AGTTGTACAA CAGGGGGCC CTCAGTCTCG TAGACGACCT TGGCCGTCTT

255 ValProGlu GlnGluMetG luValGlnG1 uProAlaGlu ProThrGlyV alaAsnMetLe userProGly GluserGluH isLeuLeuG1 uProAlaGlu

1001 GCTGAAAGGT CTCAGAGGAG GAGGTGCTG GTTCCAGCAA ATGAAGGTGA TCCCACTGAG ACTCTGAGAC AGTGTCTGGA TGACTTTGCA GACTTGGTGC  
CGACTTTCCA GAGTCTCCTC CTCGGACGAC CAAGGTCTGT TACTTCCACT AGGTGACTC TGAGACTCTG TCACGAAGCT ACTGAAACGT CTGAACCCACG

288 AlaGluArgS erGlnArgAr gArgLeuLeu ValProAlaA snGluGlyAs pProThrGlu ThrLeuArgG lnCysPheAs pAspPheAla AspLeuValPro

1101 CCTTTGACTC CTGGGAGCCG CTCATGAGGA AGTTGGGCCT CATGCACAAAT GAGATAAAGC TGGCTAAAGC TGAGGCAGCG GGCACACAGG ACACCTTCTA  
GGAAACTGAG GACCTCGGC GAGTACTCCT TCAACCCGGA GTAGCTGCTTCTGCTATCTGC AGCGATTTGG ACTCCGTCGC CCGGTGTCGC TGTGGAACAT  
322 pheAspsa xtrpGluPro LeuMetArgL yalauglyLe GCGCGCTCTA CCGGAGACAGG TGTGGGACGA CCTACGGAAC CTCTGCGAAC CTCTCTCTGA ACGGTTCGTC  
1201 CACGATGCTG ATAAAGTGGG TCAACAAAC CCGGCGAGAT GCTCTGCTCC ACACCTGCT GGATGCCTTG GAGACGCTGG GACAGAGACT TGCCAAAGCAG  
355 ThrMetLeu IleLysTrp alaAsnLysTh rGlyArgAsp AlaservAlH isthrLeuLe uAspAlaLeu CluthrLeuG lyGluArgLe uAlaLysGln  
1301 AAGATTGAGG ACCACTTGT GAGCTCTGGA AAGTTCATGT ATCTAGAAGG TAATGCAGAC TCTGCCWTGT CCTAAGTGTG ATTCCTCTTCA GGAAGTGAGA  
388 LysIleGluA spHisLeuLe userSerGly LysPheMetT yrLeuGluG1 yAsnAlaAsp SerAlaXqQs eROG\* TAGATCTTCC ATTACGTCCTG AGACGGAACA GGATTCACAC TAAGAGAAGT CCTTCACTCT  
1401 CCTTCCCTGG TTTACCTTTT TTCTGGAAAA AGCCCAACTG GACTCCAGTC AGTAGGAAAG TGCCACAATT GTCACATGAC CGGTACTGGA AGAAACTCTC  
GGAAGGGACC AAATGGAAAA AAGACCTTTT TCGGGTTGAC CTGAGGTCCG TCATCCTTTC ACGGTGTAA CAGTGTACTG GCCATGACCT TCTTTGAGAG  
1501 CCATCCAACA TCACCCAGTG CATGGAACAT CCTGTAACTT TTCACTGCAC TTGGCATTAT TTTTATAAGC TGAATGTGAT AATAAGGACA CTATGGAAAT  
GGTAGGTTGT AGTGGGTCAC CTACCTTGTA GGACATTGAA AAGTGACGTG AACCGTAATA AAAATATTTCG ACTTACACTA TTATTCCTGT GATACCTTTA  
1601 GTCTGGATCA TTCCGTTTGT GCGTACTTTG AGATTTGGTT TGGGATGTCA TTGTTTTTAC AGCAGCTTTT TATCCTAAATG TAAATGCTTT ATTTATTTAT  
CAGACCTAGT AAGGCRAACA CGCATGAAAC ACCCTACAGT AACAAAAAGTG TCGTGAAAAA ATAGGATTAC ATTTACGAAA TAAATATAATA  
1701 TTGGGCTACA TTGTAAGATC CATCTACAAA AAAAAAAA AAAAAAAA GGGGGCGCG ACTCTAGAGT CGACCTGCGC AAGCTTGCGC GCCATGGCC  
AACCCGATGT AACATTCTAG GTAGATGTTT TTTTTTTTTT TTTTTTTTTT CCGCGGCGCG TGAGATCTCA GCTGGACGTC TTCGAACCGG CCGTACCGG

Fig. 1 (cont.)

Fig. 2 A

1 MEORGONAPAAAGARKRHGPGPREARGPGLRVPKTLVLVAVALLVSAESALITQOD  
61 LAPQORAAPOQKRSSPSEGLCPPGHHISEDGRDCISCKYQDYSTHWNDLLFCRLCTRCD  
121 SGEVELSPCTTTRNTVCQCEEGTFREEDSPENCRKCRGCPRGVVKVGDCTPWSDIQVH  
181 KESGIIIGVTVAAVVLIVAVFVCKSLMKKVLPLYKIGICSGGGGDPERVDRSSQRPGEAD  
241 NVLNEIVSILQPTQVPEQEMEVEQEPAPETGVNMLSPGESEHLLLEPAEAERSQRRRLVPA  
301 NEGDPTETLRQCFDDFADLVPFDSWEPLMRKGLMDNEIKVAKAEAAAGHRDTLYTMLIKW  
361 VNKTGRDASVHTLLDLETLGERLAKQKIEDHLLSSGKFMYLEGNADSALS

Apo2  
DR4  
Apo3/DR3  
TNFR1  
Fas/Apo1

Fig. 2 B

Apo2  
DR4  
Apo3/DR3  
TNFR1  
Fas/Apo1

RA	AL	VP	ED	DS	W	E	P	L	M	R	K	E	G	L	M	D	N	E	I	K	V	A	K	A	E	A	-	-	G	H	R	D	T	H				
EA	NI	VP	ED	DS	W	D	Q	L	M	R	Q	H	D	L	T	K	A	E	I	D	V	R	A	G	T	A	-	-	G	P	G	D	A	L				
VM	HA	VP	AR	RR	RR	RR	RR	RR	RR	RR	T	D	G	R	E	A	E	T	E	A	L	E	V	E	I	G	R	-	-	F	H	D	Q	Q				
VV	EN	VP	PL	RR	RR	RR	RR	RR	RR	RR	H	G	L	S	D	H	E	I	D	R	L	E	L	Q	N	G	R	-	C	L	E	E	A	Q				
IG	VM	TL	S	Q	V	K	G	E	V	R	K	N	G	V	N	E	A	K	I	D	E	I	K	N	D	A	V	Q	D	T	A	E	Q	K	V			
TM	IG	VM	VN	K	T	C	R	D	-	A	S	V	H	T	L	L	D	A	L	E	T	L	G	E	E	L	A	N	Q	K	I	E	D					
Y	A	M	L	K	R	M	VN	K	T	C	R	N	-	A	S	I	H	T	L	L	D	A	L	E	R	M	E	E	H	A	K	E	K	I	Q	D		
Y	E	M	L	K	R	M	R	Q	Q	P	-	A	G	L	G	A	V	Y	A	L	E	R	M	G	L	D	C	V	E	D	L	R	S					
Y	S	E	L	A	T	M	R	R	R	P	R	R	E	A	T	L	E	I	G	R	V	H	R	D	M	D	L	L	C	L	E	D	I	E				
-	Q	L	L	R	N	N	H	Q	L	H	C	K	K	E	A	Y	-	D	T	H	I	K	D	H	K	K	A	N	G	C	T	L	A	E	K	I	Q	T



Fig. 3

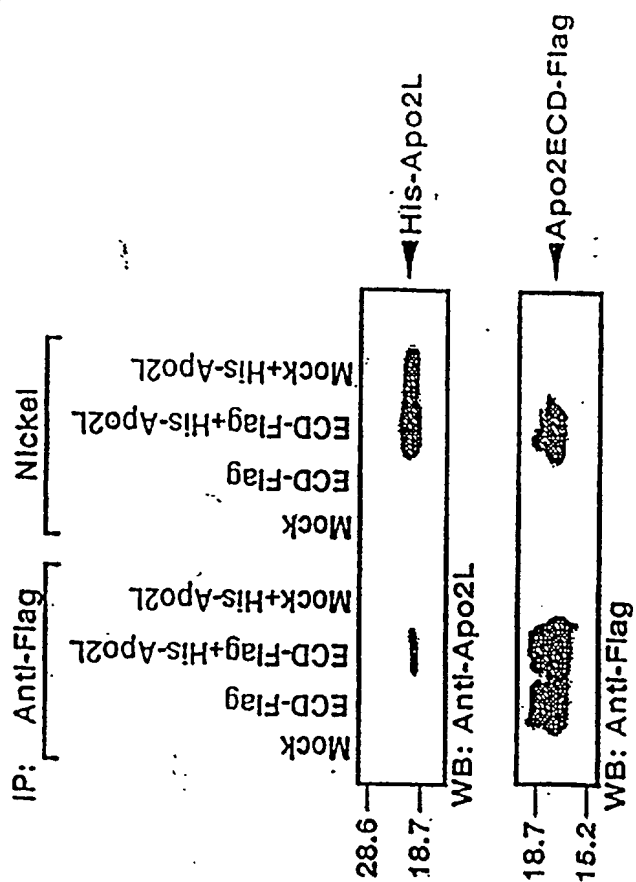
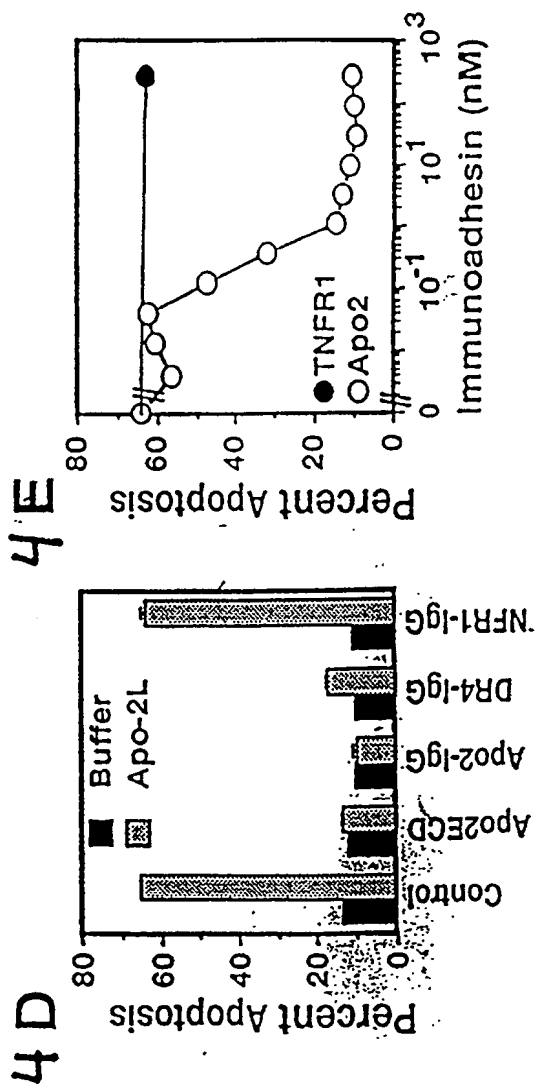
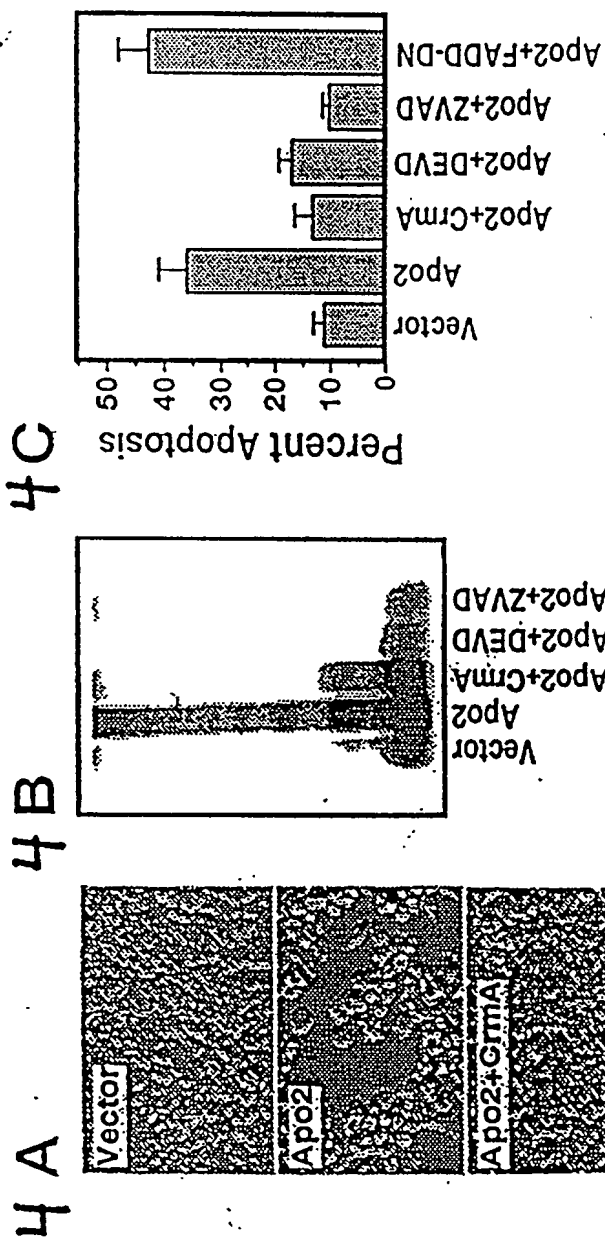


Fig. 4



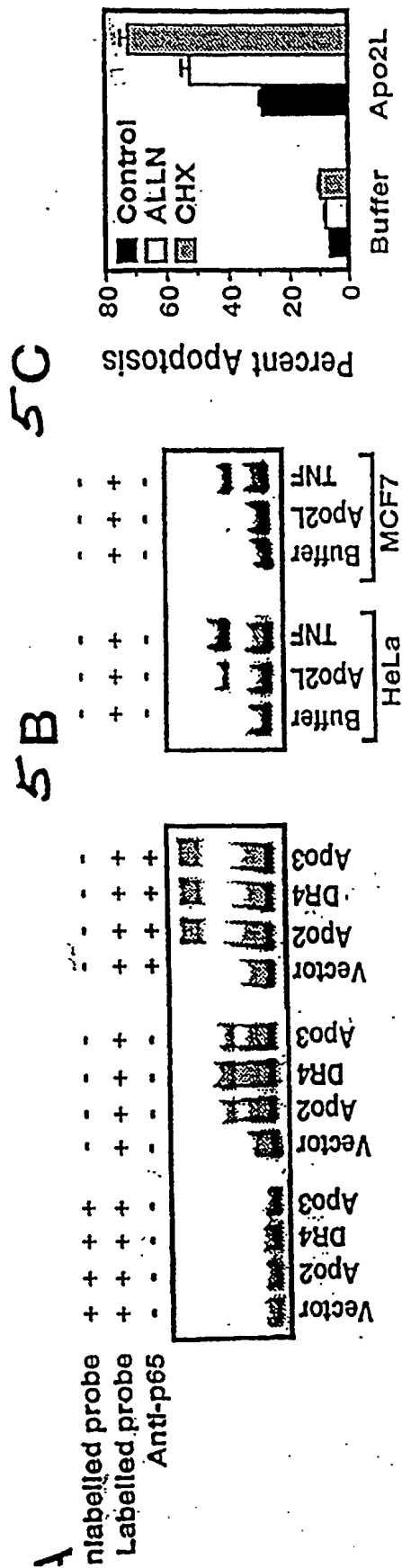
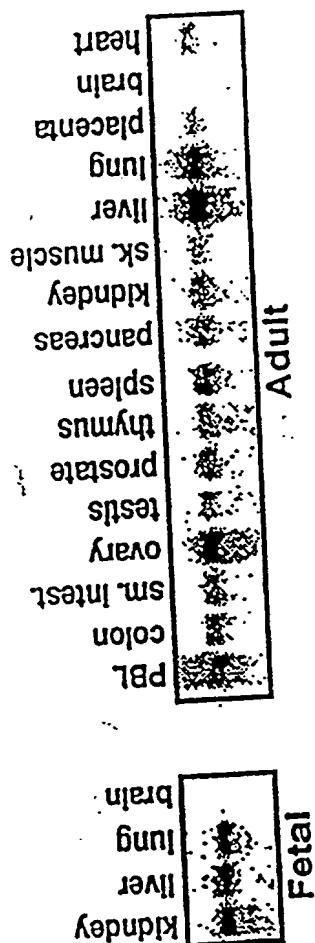


FIG. 5

FIG. 6A



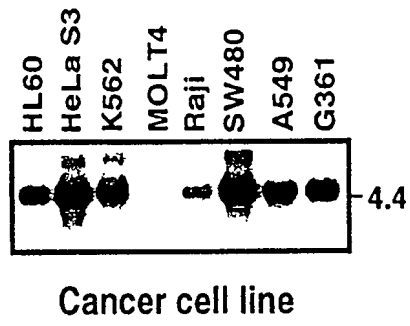


Fig. 6B

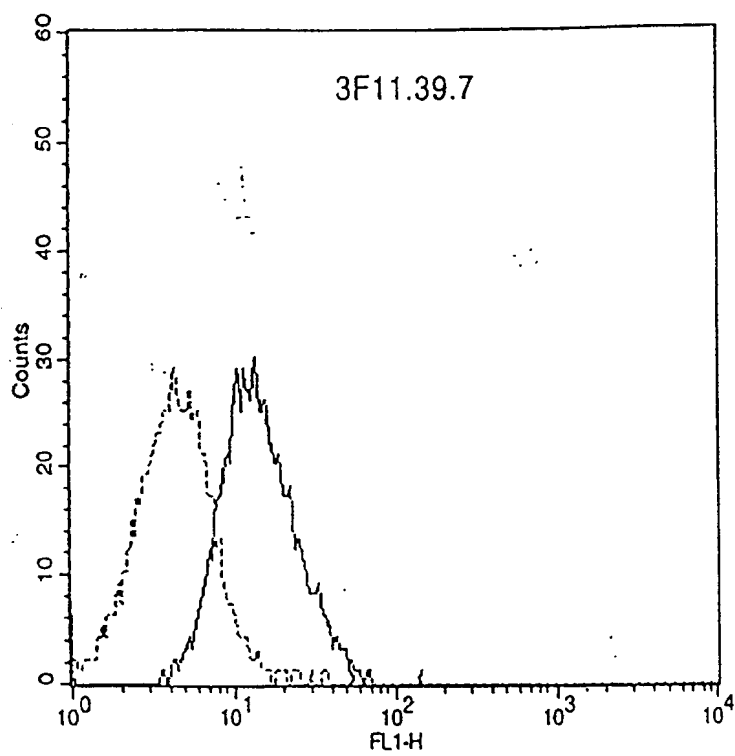


Fig. 7

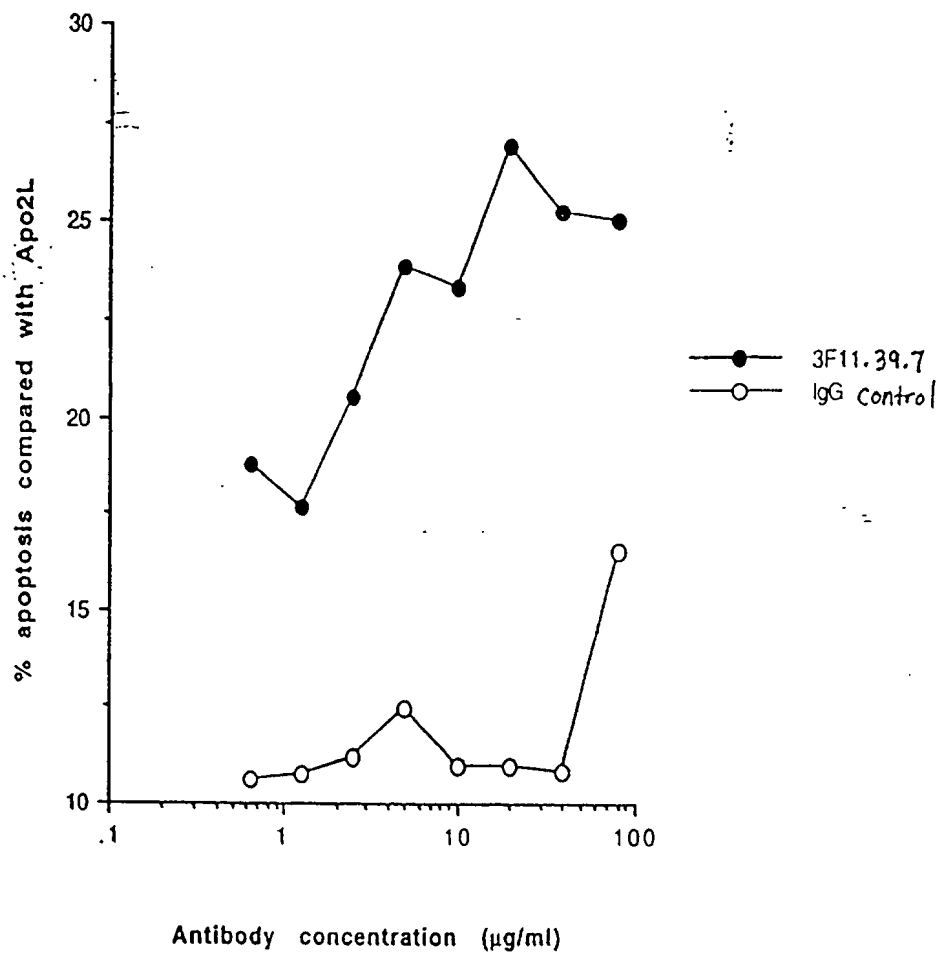


Fig. 8

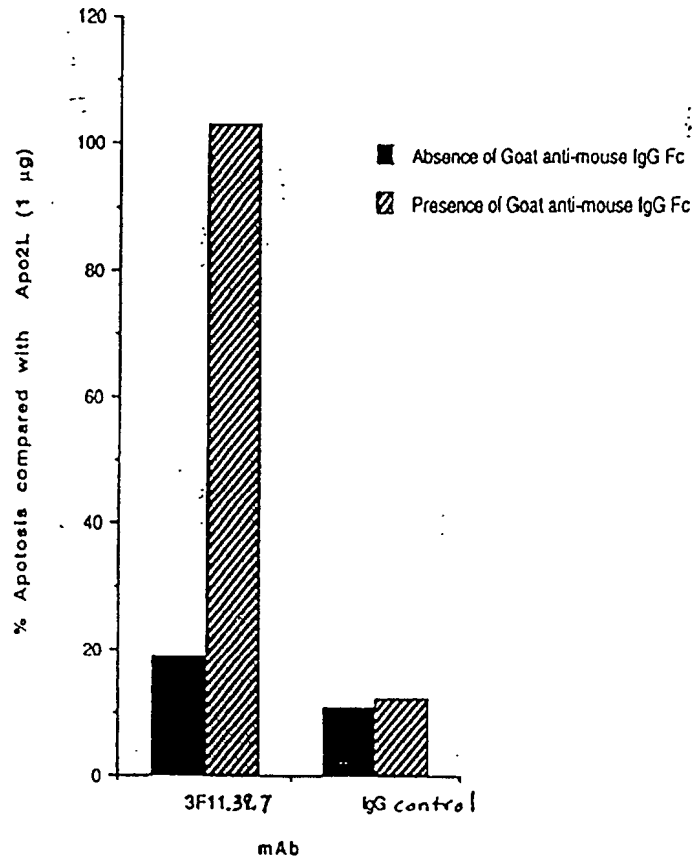


Fig. 9



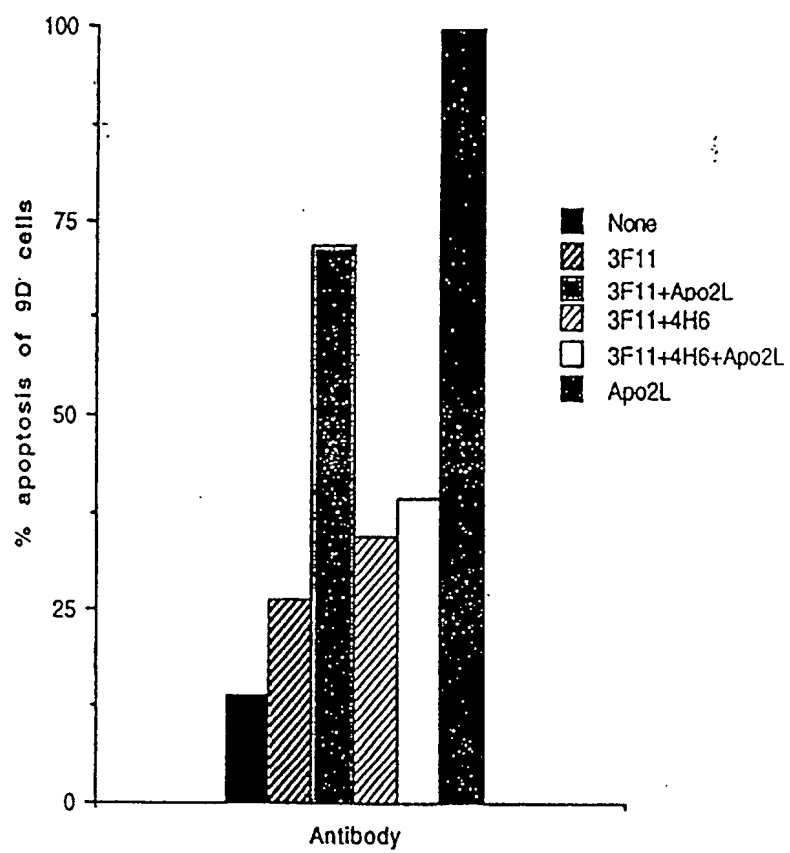


Fig: 10

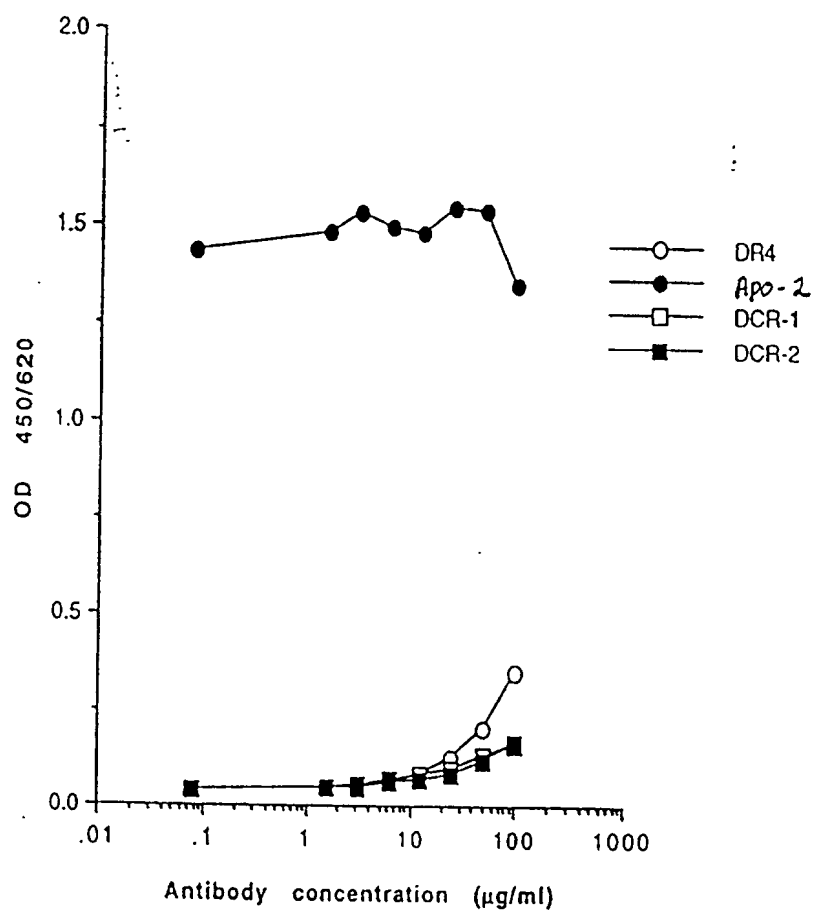


Fig. 11

anti-Apo-2 16E2-his

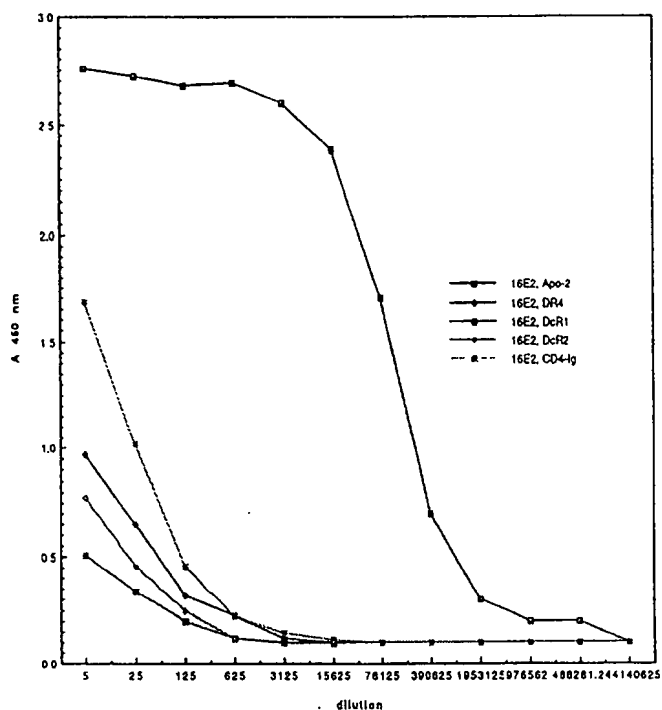


Fig. 12A

anti-Apo-2 20E6-his

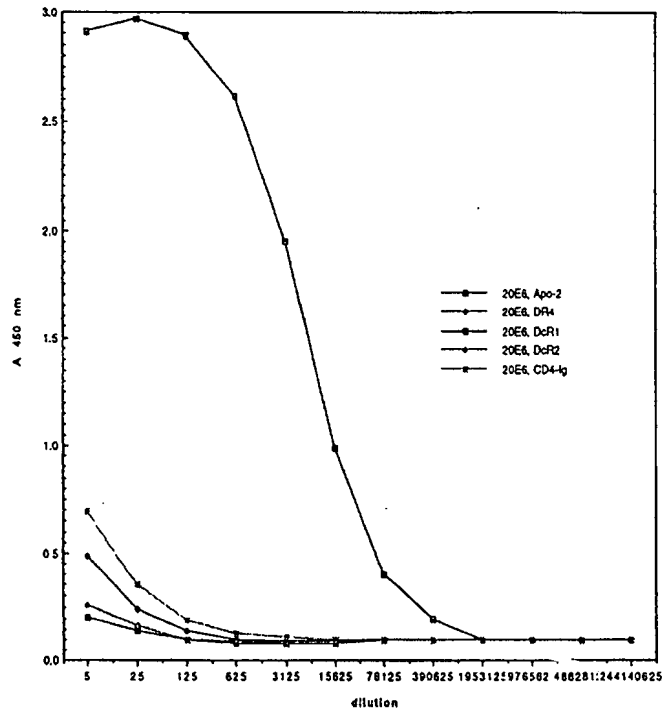


Fig. 12B

102011-362500

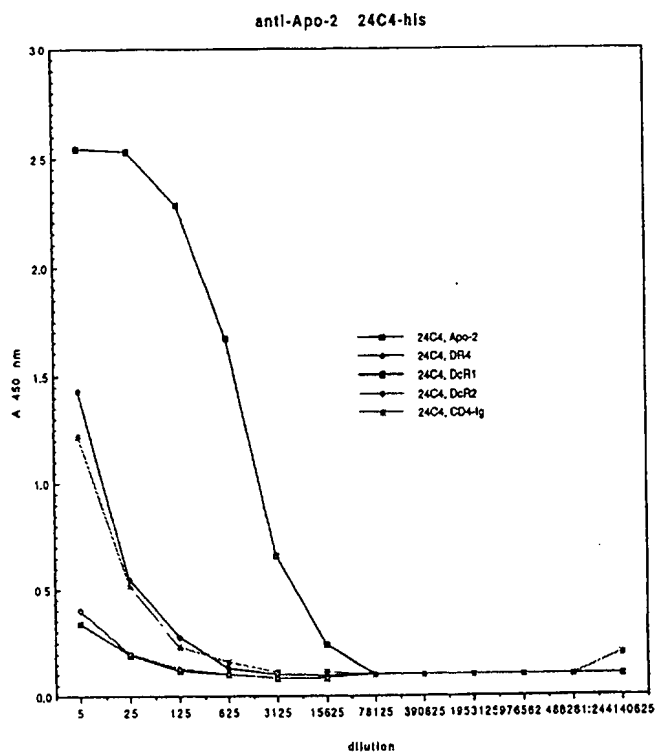


Fig. 12c

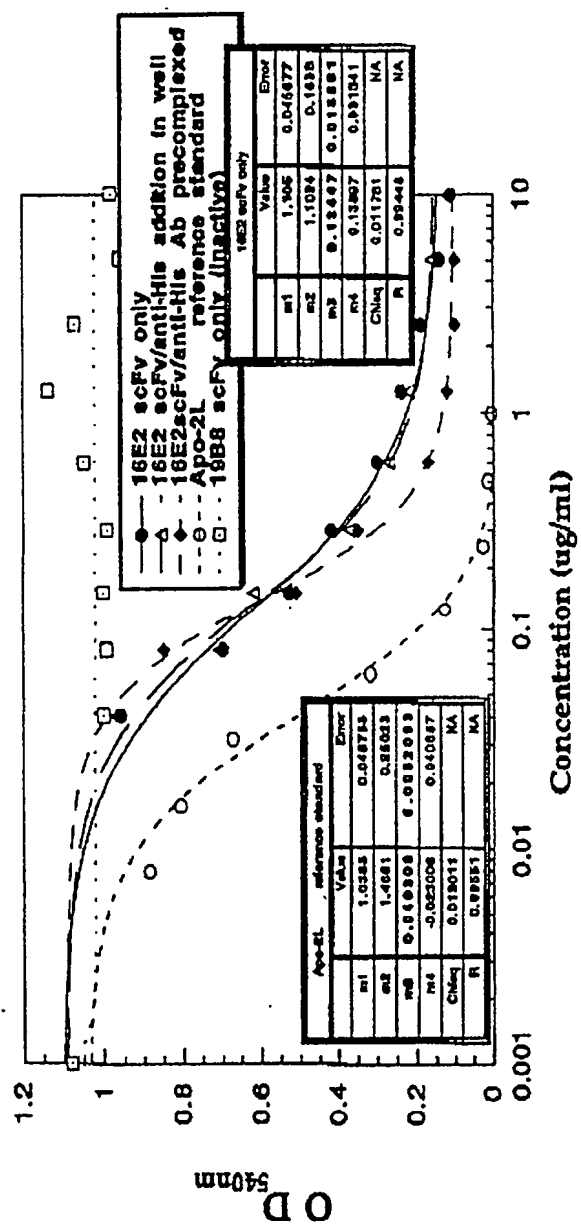


Fig. 13A

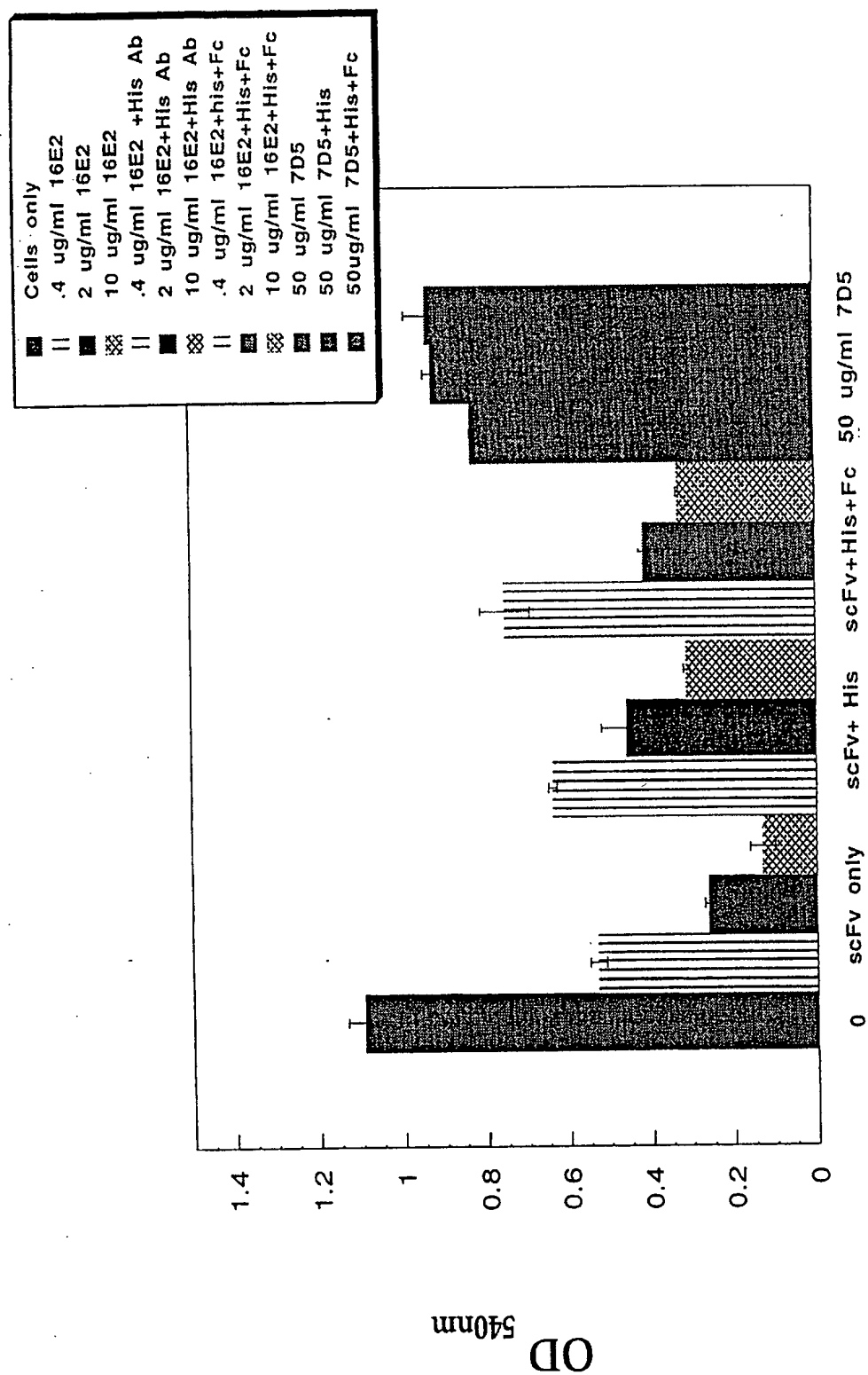


Fig. 13B

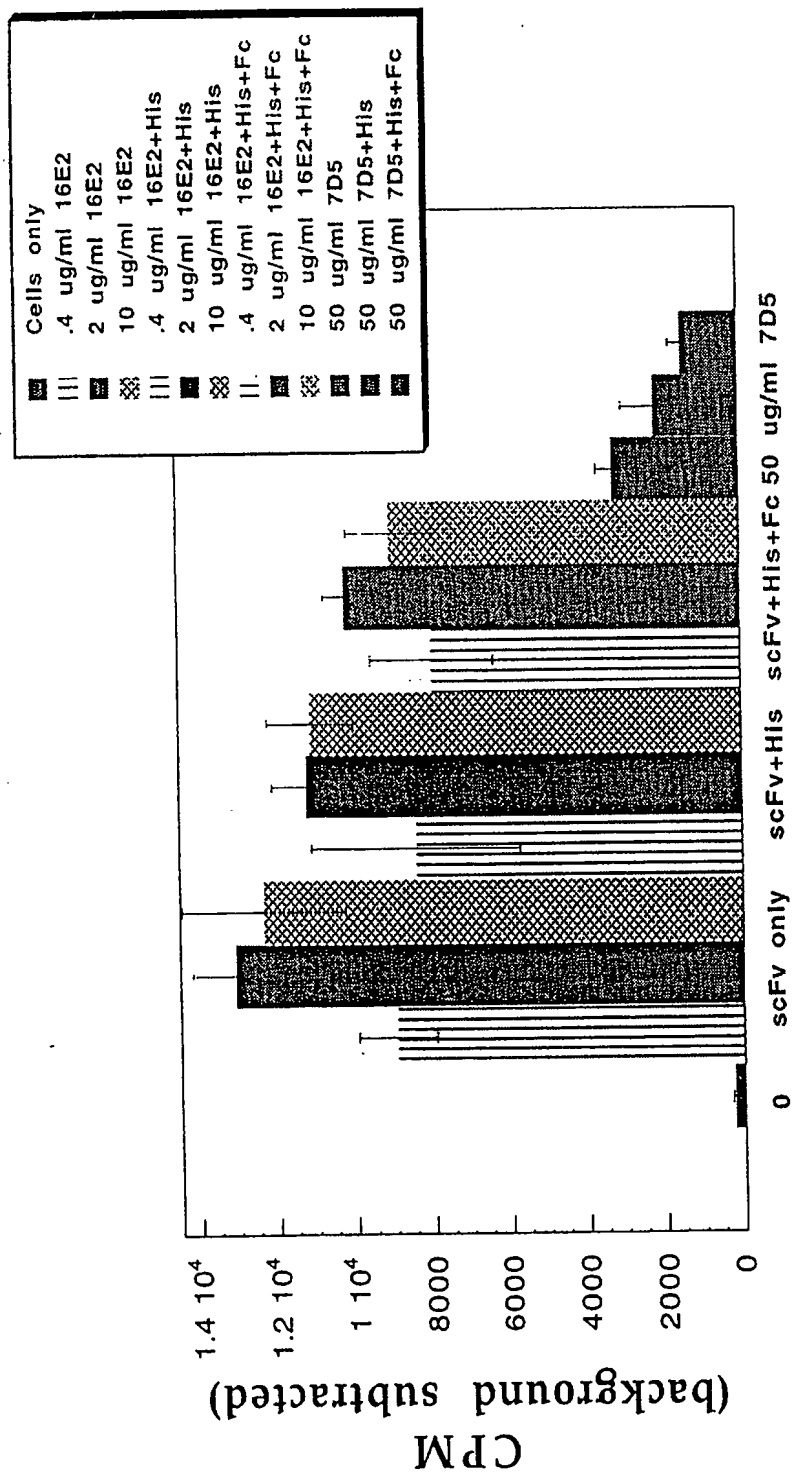


Fig. 13c



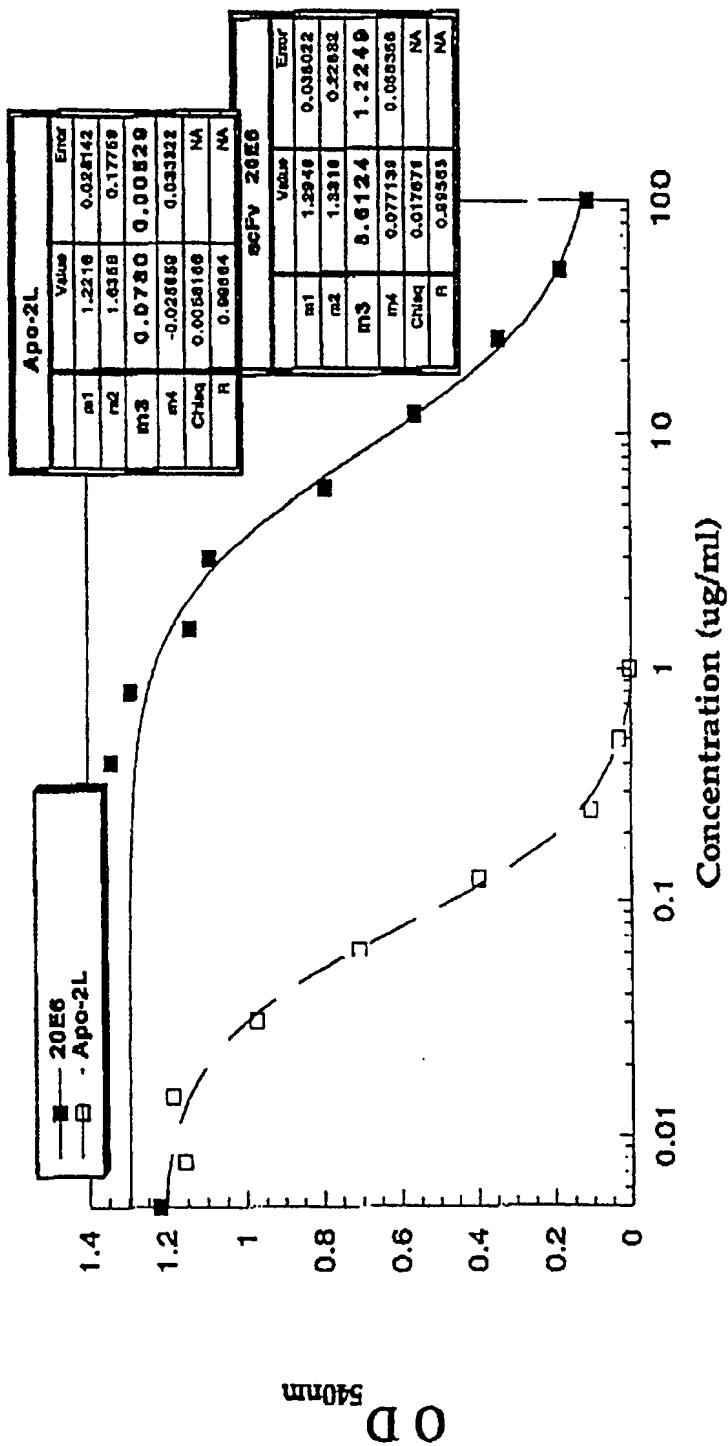


Fig 14A

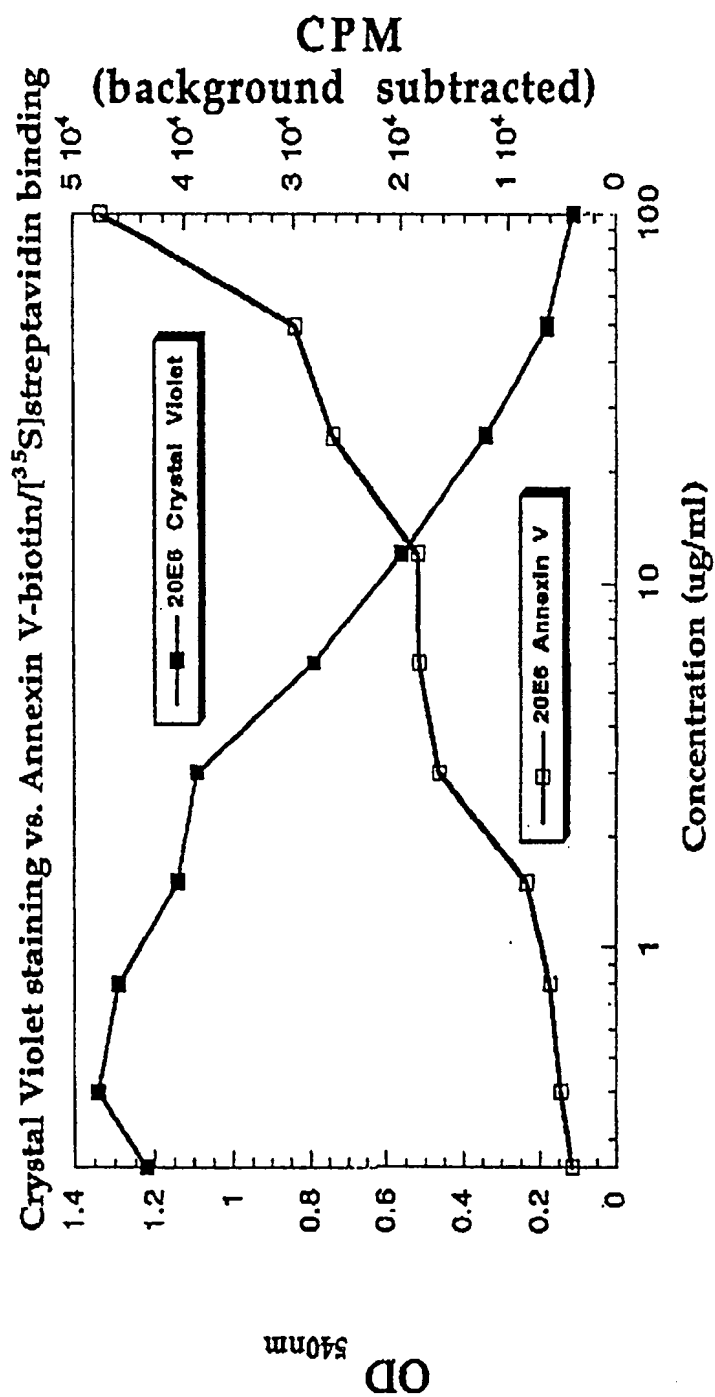


Fig. 14B

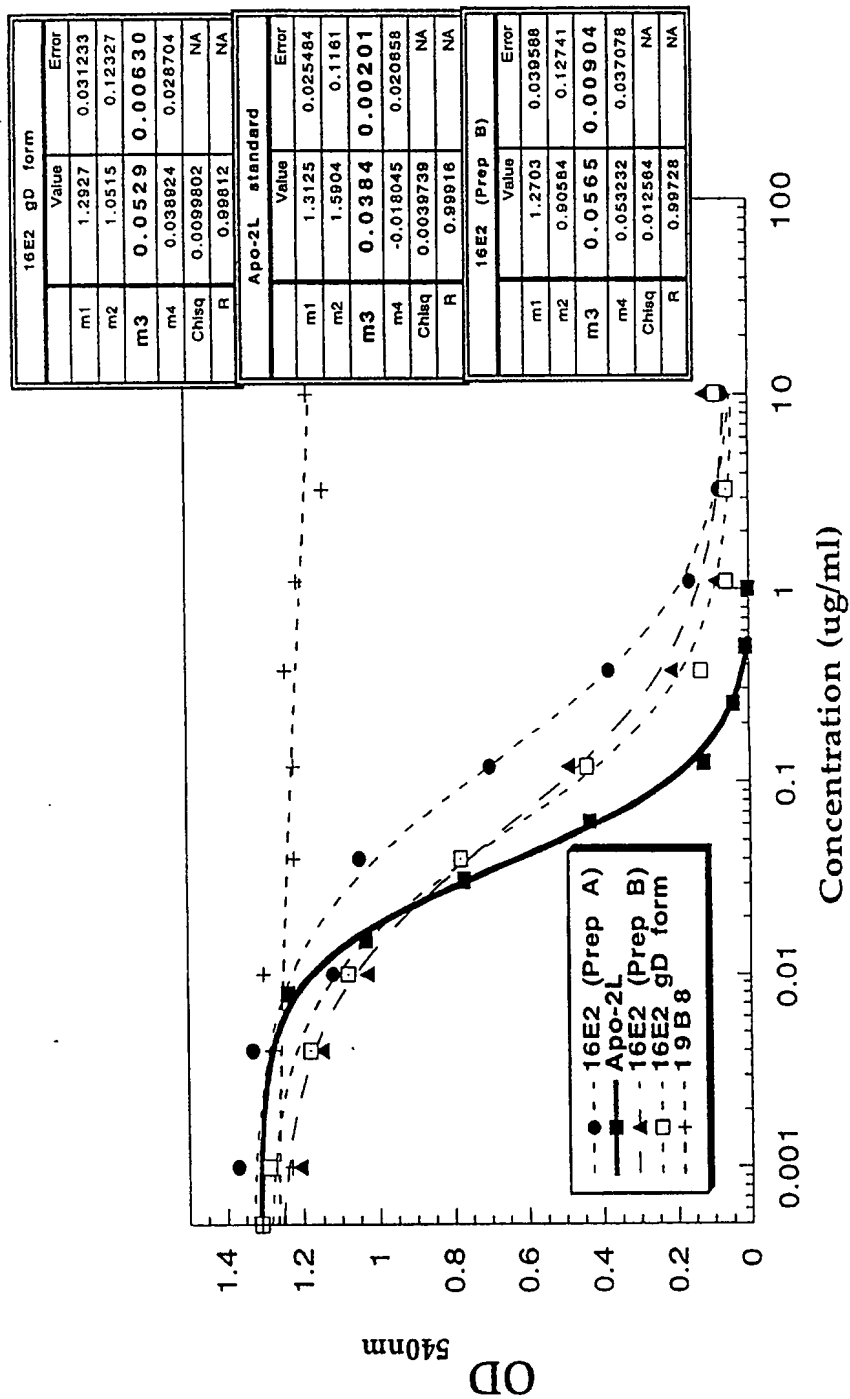


Fig. 14C

ATGACCATGA TTACGCCAAG CTTTGGAGCC TTTTTTTTGG AGATTTTCAA 50  
 CGTGAAAAAA TTATTATTCG CAATTCCTTT AGTTGTTTCCT TTCTATGCGG 100  
 CCCAGCCGGC CATGGCCGAG GTGCAGCTGG TGCAGTCTGG GGGAGGTGTG 150  
 GAACGGCCGG GGGGGTCCCT GAGACTCTCC TGTGCAGCCT CTGGATTCAC 200  
 CTTTGATGAT TATGGCATGA GCTGGGTCCG CCAAGCTCCA GGAAGGGGGC 250  
 TGGAGTGGGT CTCTGGTATT AATTGGAATG GTGGTAGCAC AGGATATGCA 300  
 GACTCTGTGA AGGGCCGAGT CACCATCTCC AGAGACAACG CCAAGAACTC 350  
 CCTGTATCTG CAAATGAACA GCCTGAGAGC CGAGGACACG GCCGTATATT 400  
 ACTGTGCGAA AATCCTGGGT GCCGGACGGG GCTGGTACTT CGATCTCTGG 450  
 GGAAGGGGA CCACGGTCAC CGTCTCGAGT GGTGGAGGCG GTTCAGGCGG 500  
 AGGTGGCAGC GGCGGTGGCG GATCGTCTGA GCTGACTCAG GACCCTGCTG 550  
 TGTCTGTGGC CTTGGGACAG ACAGTCAGGA TCACATGCCA AGGAGACAGC 600  
 CTCAGAAGCT ATTATGCAAG CTGGTACCAG CAGAAGCCAG GACAGGCCCC 650  
 TGTACTTGTC ATCTATGGTA AAAACAACCG GCCCTCAGGG ATCCCAGACC 700  
 GATTCTCTGG CTCCAGCTCA GGAAACACAG CTTCTTGAC CATCACTGGG 750  
 GCTCAGGCGG AAGATGAGGC TGA CTATTAC TGTA ACTCCC GGGACAGCAG 800  
 TGGTAACCAT GTGGTATTCG GCGGAGGGAC CAAGCTGACC GTCCTAGGTG 850  
 CGGCCGCACA TCATCATCAC CATCACGGGG CCGCAGAACA AAAACTCATC 900  
 TCAGAAGAGG ATCTGAATGG GGCCGCATAG 930

Fig. 15A

ATGACCATGA TTACGCCAAG CTTTGGAGCC TTTT'TTTTGG AGATTTTCAA 50  
 CGTGAAAAAA TTATTATTCG CAATTCCTTT AGTTGTTTCCT TTCTATGCGG 100  
 CCCAGCCGGC CATGGCCGGG GTGCAGCTGG TGGAGTCTGG GGGAGGCTTG 150  
 GTCCAGCCTG GGGGGTCCCT GAGACTCTCC TGTGCAGCCT CTGGATTAC 200  
 CTTTAGTAGC TATTGGATGA GCTGGGTCCG CCAGGCTCCA GGAAGGGGC 250  
 TGGAGTGGGT GGCCAACATA AAGCAAGATG GAAGTGAGAA ATACTATGTG 300  
 GACTCTGTGA AGGGCCGATT CACCATCTCC AGAGACAACG CCAAGAACTC 350  
 ACTGTATCTG CAAATGAACA GCCTGAGAGC CGAGGACACG GCTGTGTATT 400  
 ACTGTGCGAG AGATCTTTTA AAGGTCAAGG GCAGCTCGTC TGGGTGGTTC 450  
 GACCCCTGGG GGAGAGGGAC CACGGTCACC GTCTCGAGTG GTGGAGGCGG 500  
 TTCAGGCGGA GGTGGTAGCG GCGGTGGCGG ATCGTCTGAG CTGACTCAGG 550  
 ACCCTGCTGT GTCTGTGGCC TTGGGACAGA CAGTCAGGAT CACATGCCAA 600  
 GGAGACAGCC TCAGAAGCTA TTATGCAAGC TGGTACCAGC AGAAGCCAGG 650  
 ACAGGCCCCCT GTACTTGTCA TCTATGGTAA AAACAACCGG CCCTCAGGGA 700  
 TCCCAGACCG ATTCTCTGGC TCCAGCTCAG GAAACACAGC TTCCTTGACC 750  
 ATCACTGGGG CTCAGGCGGA AGATGAGGCT GACTATTACT GTAACCTCCG 800  
 GGACAGCAGT GGTAACCATG TGGTATTCGG CGGAGGGACC AAGCTGACCG 850  
 TCCTAGGTGC GGCCGCACAT CATCATCACC ATCACGGGGC CGCAGAACAA 900  
 AAACTCATCT CAGAAGAGGA TCTGAATGGG GCCGCATAG 939

Fig. 15B

ATGACCATGA TTACGCCAAG CTTTGGAGCC TTTTTTTTGG AGATTTTCAA 50  
 CGTGAAAAAA TTATTATTCG CAATTCCTTT AGTTGTTCCCT TTCTATGCGG 100  
 CCCAGCCGGC CATGGCCCAG GTGCAGCTGG TGCAGTCTGG GGGAGGCGTG 150  
 GTCCAGCCTG GGCGGTCCCT GAGACTCTCC TGTGCAGCTT CTGGGTTCAT 200  
 TTTCACTAGT TATGGGATGC ACTGGGTCCG CCAGGCTCCA GGCAAGGGGC 250  
 TGGAGTGGGT GGCAGGTATT TTTTATGATG GAGGTAATAA ATACTATGCA 300  
 GACTCCGTGA AGGGCCGATT CACCATCTCC AGAGACAATT CCAAGAACAC 350  
 GCTGTATCTG CAAATGAACA GCCTGAGAGC TGAGGACACG GCTGTGTATT 400  
 ACTGTGCGAG AGATAGGGGC TACTACTACA TGGACGTCTG GGGCAAAGGG 450  
 ACCACGGTCA CCGTCTCCTC AGGTGGAGGC GGTTCAGGCG GAGGTGGCTC 500  
 TGGCGGTGGC GGATCGCAGT CTGTGTTGAC GCAGCCGCCC TCAGTGTCTG 550  
 GGGCCCCAGG ACAGAGGGTC ACCATCTCCT GCACTGGGAG AAGCTCCAAC 600  
 ATCGGGGCAG GTCATGATGT ACACTGGTAC CAGCAACTTC CAGGAACAGC 650  
 CCCCAACTC CTCATCTATG ATGACAGCAA TCGGCCCTCA GGGGTCCCTG 700  
 ACCGATTCTC TGGCTCCAGG TCTGGCACCT CAGCCTCCCT GGCCATCACT 750  
 GGGCTCCAGG CTGAAGATGA GGCTGATTAT TACTGCCAGT CCTATGACAG 800  
 CAGCCTGAGG GGTTCGGTAT TCGGCGGAGG GACCAAGGTC ACTGTCCTAG 850  
 GTGCGGCCGC ACATCATCAT CACCATCACG GGGCCGCAGA AAAAAAATC 900  
 ATCTCAGAAG AGGATCTGAA TGGGGCCGCA TAG 933

Fig. 15C

	signal	Heavy chain
Apo-2.16E2.his	1	MIMITPSFGAFFLEIFNVKKLLFAIPLVVPFYAAQPAMAEVQLVQSGGGV
Apo-2.20E6.his	1	MIMITPSFGAFFLEIFNVKKLLFAIPLVVPFYAAQPAMAGVQLVESGGGL
Apo-2.24C4.his	1	MIMITPSFGAFFLEIFNVKKLLFAIPLVVPFYAAQPMAGVQLVQSGGGV

	CDR1	CDR2
Apo-2.16E2.his	51	ERPGGSLRLSCAASGFTFDDYGM <del>SWRQAPGKGLEWVSGINMNGGSTGYA</del>
Apo-2.20E6.his	51	VQPGGSLRLSCAASGFTFSSY <del>MSWRQAPGKGLEWVANIKODGSEKYYV</del>
Apo-2.24C4.his	51	VQPGSLRLSCAASGFTFSSY <del>GMHWRQAPGKGLEWVAGIFYDGGNKYYA</del>

	CDR3
Apo-2.16E2.his	101 <u>DSVKGRVTISRDN</u> AKNSLYLQ <del>MNSLRAEDTAVYYCAK IL----</del> GAGRGWY
Apo-2.20E6.his	101 <u>DSVKGRFTISRDN</u> AKNSLYLQ <del>MNSLRAEDTAVYYCARDLLKVKGSSSGW-</del>
Apo-2.24C4.his	101 <u>DSVKGRFTISRDN</u> SKNTLYLQ <del>MNSLRAEDTAVYYCARD-----</del> RGYY

	Light chain
Apo-2.16E2.his	147 <u>F-DLWKG</u> GTTIVTVSSGGGGSGGGGSGGGGS-SELTQDPAVSVALGQIVRI
Apo-2.20E6.his	150 <u>F-DFWGR</u> GTTIVTVSSGGGGSGGGGSGGGGS-SELTQDPAVSVALGQIVRI
Apo-2.24C4.his	143 <u>YMDVW</u> GKGTTIVTVSSGGGGSGGGGSGGGGSQSVLTQPPSVSGAPGQRVTI

	CDR1	CDR2
Apo-2.16E2.his	195 TC <u>CGDSL</u> R---SY <del>YASWYQQKPGQAPVLVTY</del> GKNNRPSGIPDRFSGSSSG	
Apo-2.20E6.his	198 TC <u>CGDSL</u> R---SY <del>YASWYQQKPGQAPVLVTY</del> GKNNRPSGIPDRFSGSSSG	
Apo-2.24C4.his	193 SC <u>TGRSS</u> NIGAGH <del>LVHWYQQLPGTAPKLLTY</del> DDSNRPSGVPDRFSGSRSG	

	CDR3
Apo-2.16E2.his	242 NTASLTTTGAQAED <del>EADYYCNSRDSSGNHV</del> FGGGTKLTVLGAAAHHHH
Apo-2.20E6.his	245 NTASLTTTGAQAED <del>EADYYCNSRDSSGNHV</del> FGGGTKLTVLGAAAHHHH
Apo-2.24C4.his	243 TSASLAITGLQAED <del>EADYYCQSYDSSLRGSV</del> FGGGTKVTVLGAAAHHHH

Apo-2.16E2.his	292 HGAAEQKLISEEDLN <del>GAA</del>
Apo-2.20E6.his	295 HGAAEQKLISEEDLN <del>GAA</del>
Apo-2.24C4.his	293 HGAAEQKLISEEDLN <del>GAA</del>

Fig. 16